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Decreases in Cocaine Self Administration with Dual Inhibition of the Dopamine Transporter and σ Receptors

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Running title page

Running title: Dual DAT/σR Blockade and Cocaine Self-Administration

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Non-standard abbreviations:

DTG: 1,3-di-(2-tolyl)guanidine

BD 1008: N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(1-pyrrolidinyl)ethylamine dihydrobromide

BD 1047: N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino) ethylamine dihydrobromide

BD 1063: N-[2-(3,4-dichlorophenyl) ethyl]-4-methylpiperazine dihydrochloride

NE-100: N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ethylamine monohydrochloride

AC927: N-phenethylpiperidine oxalate

Rimcazole: 9-[3-(cis-3,5-Dimethyl-1-piperazinyl)propyl]-9H-carbazole dihydrochloride

SH 3-24: [3-(cis-3,5-dimethyl-4-[3-phenylpropyl]-1-piperazinyl)-propyl]diphenylamine hydrochloride

SH 3-28: 9-[3-(cis-3,5-dimethyl-4-[3-phenylpropyl]-1-piperazinyl)-propyl]carbazole hydrobromide

σR: Sigma receptor

σ_1R: Sigma$_1$ receptor

σ_2R: Sigma$_2$ receptor

DAT: Dopamine Transporter

ABSTRACT

Sigma receptor (σR) antagonists attenuate many behavioral effects of cocaine, but typically not its reinforcing effects in self-administration procedures. However, the σR antagonist rimcazole and its *N*-propylphenyl analogs, SH 3-24 and SH 3-28, dose-dependently decreased the maximal rates of cocaine self administration without affecting comparable responding maintained by food reinforcement. In contrast, a variety of σR antagonists (AC927, BD 1008, BD 1047, BD 1063, NE-100) had no effect on cocaine self-administration across the range of doses that decreased rates of food-maintained responding. Rimcazole analogs differed from selective σR antagonists in their dual affinities for σRs and the dopamine transporter (DAT) assessed with radioligand binding. Selective DAT inhibitors and σR antagonists were studied alone and in combination on cocaine self-administration to determine whether actions at both σRs and the DAT were sufficient to reproduce the effects of rimcazole analogs. Typical DAT inhibitors (WIN 35,428, methylphenidate, nomifensine) dose-dependently shifted the cocaine dose-effect curve leftward. Combinations of DAT-inhibitor and σR-antagonists doses that were behaviorally inactive alone decreased cocaine self-administration without effects on food-maintained responding. Additionally, whereas the DAT inhibitors were self-administered at rates similar to those of cocaine, neither rimcazole analogs nor typical σR antagonists (NE-100, AC927) maintained responding above control levels across a wide range of doses. These findings suggest that unique effects of rimcazole analogs are due to dual actions at the DAT and σRs, and that a combined target approach may have utility in development of medical treatments for cocaine abuse.

INTRODUCTION

Several studies have demonstrated that σ receptor (σR) antagonists block some behavioral effects of cocaine. For example, pretreatment with σR antagonists, α-(4-fluorophenyl)-4-(5-fluoro-2-pyrimidinyl)-1-piperazine-butanol (BMY 14802) or 9-[3-(cis-3,5-dimethyl-1-piperazinyl)propyl]-9H-carbazole dihydrochloride (rimcazole), antagonized locomotor stimulatory effects of cocaine in mice (Menkel et al., 1991). In addition, several σR antagonists significantly attenuated the development of stimulant-induced locomotor sensitization (Ujike et al., 1992; Witkin et al., 1993). Further, pretreatment with a variety of σR antagonists reduces acute toxic effects of cocaine, including convulsions and lethality (for reviews see Matsumoto, 2009; Maurice and Su, 2009).

Despite the blockade of locomotor stimulation and acute toxic effects of cocaine by a variety of σR antagonists, studies on blockade of effects of cocaine related to reinforcement have reported inconsistent outcomes. Pretreatments with σR antagonists *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine dihydrobromide (BD 1047) or N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ethylamine monohydrochloride (NE-100) decreased cocaine-induced place conditioning in mice (Romieu et al., 2002; Romieu et al., 2004). However, an initial report showed that a selected dose of BD 1047 on cocaine self-administration in rats was inactive (Martin-Fardon et al., 2007). A subsequent study that examined a wide range of doses of BD 1047 and its analogs (BD 1008, BD 1063) similarly found a lack of effects on self administration of a broad range of cocaine doses (Hiranita et al., 2010).

The mechanism by which σR antagonists block some of the effects of cocaine has not been determined. A number of studies have suggested interactions between σR and dopamine

systems at which cocaine appears to act predominantly to produce its behavioral effects. For example, Wachtel and White (1988) showed that BMY 14802 reversed the suppressant effects of apomorphine on the firing of both A9 and A10 dopamine neurons. Further, Gonzalez-Alvear and Werling (1994) showed a σR-mediated modulation of dopamine release. More recently, Su and colleagues (e.g. Hayashi et al., 2000) showed that σRs are linked to intracellular calcium signaling mechanisms that may alter short-term or long-term effects of cocaine (Su and Hayashi, 2001). With an extensive array of intracellular functions of σRs there are many potential mechanisms that may contribute to the interactions of stimulants and σR ligands (Katz et al., 2011).

Matsumoto et al. (2001) suggested that the interaction between cocaine and σR ligands is a competitive antagonism of effects of cocaine mediated by σRs. On the other hand, Izenwasser et al. (1993) found that several σR ligands, including rimcazole, inhibited dopamine uptake and had μmolar affinity for the DAT. Thus, some σR ligands may alter the effects of cocaine through an action at the DAT. Consistent with this interpretation, a recent finding (Loland et al., 2008) showed that rimcazole analogs bind to the DAT in a manner favoring a DAT conformation that renders it less accessible to the extracellular space. In contrast, cocaine analogs bind to the DAT in a manner that favors an outward facing conformation (Reith et al., 2001; Loland et al., 2008).

Rimcazole and two of its analogs [3-(cis-3,5-dimethyl-4-[3-phenylpropyl]-1-piperazinyl)-propyl]diphenylamine hydrochloride (SH 3-24) or 9-[3-(cis-3,5-dimethyl-4-[3-phenylpropyl]-1-piperazinyl)-propyl]carbazole hydrobromide (SH 3-28) (Husbands et al., 1999) were reported to block the locomotor stimulant effects of cocaine at doses that were themselves behaviorally inactive (Menkel et al., 1991; Katz et al., 2003). These findings coupled with those of Loland et

al. (2008), suggest that rimcazole and its analogs may promote a conformational change in the DAT that disfavors cocaine binding and contributes to a distinctive behavioral profile. Therefore, the interaction of rimcazole and its analogs with cocaine may differ from that of other σR antagonists due to their dual actions. As such, rimcazole analogs in contrast to selective σR antagonists, may more effectively block cocaine self administration. Thus, the present study assessed the activity of rimcazole and two of its analogs in rats self administering cocaine, and their own abuse liability. Each of the compounds decreased rates of responding maintained by cocaine at doses that had no effects on responding maintained by food reinforcement. Because the compounds have affinity for the DAT as well as σRs, we subsequently assessed whether dual actions at these sites contributes to the blockade of cocaine self administration through combinations of compounds selective for these sites.

METHODS

Subjects. Thirty-eight male Sprague-Dawley rats (weighing approximately 300 g at the start of the study), obtained from Charles River Laboratories (Wilmington, MA), served as subjects after acclimation to the laboratory for at least one week. Some of these subjects had been used previously in a study using similar behavioral and pharmacological procedures (Hiranita et al., 2010). Food (Scored Bacon Lover Treats, BIOSERV, Frenchtown, NJ) and tap water were available in their home cages. After acclimation, weights of rats were maintained at approximately 320 g by adjusting their daily food ration. The animal housing room was temperature- and humidity-controlled and maintained on a 12:12-h light:dark cycle with lights on at 07:00 hours. Care of the subjects was in accordance with the guidelines of the National 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.

Apparatus. Experimental sessions were conducted with subjects placed in operant-conditioning chambers (modified ENV-008CT, Med Associates, St. Albans, VT) that measured 25.5 cm x 32.0 cm x 25.0 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 20 g defined a response, which 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 above each lever. A receptacle for the delivery of food pellets was mounted behind a 5.0 x 5.0 cm opening in the front wall midline between the two levers and 2.0 cm above the floor. When called for a pellet dispenser (ENV-203, Med Associates) delivered 45 mg food pellets to the receptacle. A syringe driver (Model 22, Harvard Apparatus, Holliston, MA) placed above each chamber delivered injections of specified volumes and durations from a 10 ml syringe. The syringe was connected by Tygon tubing to a single-channel fluid swivel (375 Series Single Channel Swivels, 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.

Procedures. Subjects were placed in chambers during experimental sessions that were conducted daily, seven days per week. During sessions subjects were trained with food reinforcement (45 mg food pellets, BIOSERV, Frenchtown, NJ) to press the right lever, and were subsequently trained under a fixed-ratio (FR) 5-response schedule of reinforcement (each fifth response produced a food pellet). Food deliveries were followed by a 20-sec timeout (TO) period during which all lights were off and responses had no scheduled consequences other than

the feedback click. During this training, sessions lasted for 20 min or until 30 food pellets were delivered.

After subjects were responding at a rate sufficiently high that they obtained 30 food pellets within each of three consecutive sessions they were divided into two groups. One group continued with food reinforcement, whereas subjects in the other group were surgically implanted in the right or left external jugular vein with a chronic indwelling catheter that exited at the mid-scapular region of the animal's back. Catheter implantation was performed under anesthesia (ketamine 60.0 mg/kg, i.p. and xylazine 12.0 mg/kg, i.p.). Catheters were infused daily with 0.1 ml of a sterile saline solution containing heparin (30.0 IU/ml), 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 cocaine self-administration studies were initiated.

Cocaine self-administration sessions were conducted in 2-hr daily sessions until the response rates and patterns of responding showed no substantial session to session trends. During these sessions, the LEDs above the right lever were illuminated when cocaine injections were available. Completion of five responses turned off the LEDs and activated the infusion pump, delivering a dose of 1.0 mg/kg. A 20-sec TO, during which LEDs were off and responses produced only feedback clicks, started with the injection. After the time out, the LEDs were illuminated and responding again had scheduled consequences. Once rates of responding maintained by cocaine were stable across sessions, the session was divided into five 20-min components, each preceded by a 2-min TO. This arrangement allowed the assessment of a different cocaine dose within each component. By adjusting infusion volumes and durations, the cocaine dose per injection was incremented in the five sequential components in an ascending

order as follows: no injection (also referred to as extinction, or EXT, because responses had no scheduled consequences other than turning off the LEDs for 20 sec), 0.03, 0.10, 0.32, and 1.0 mg/kg/inj. Infusion volumes and durations were respectively 0, 5.6, 18.0, 56.0, 180 µl and 0, 0.32, 1.0, 3.2 10.0 sec, based on a body weight of 0.32 kg. A response-independent "sample" injection of cocaine at the corresponding dose was administered immediately before each component.

Training continued until: 1) at least 5.0 mg/kg of cocaine was self-administered within a session with less than 20% variation in the total number of cocaine injections compared to the previous session; 2) the dose of cocaine that maintained maximal response rates varied by no more than one-half log unit over two consecutive test sessions; and 3) maximum response rates were at least five-fold higher than response rates maintained during EXT. The effects of substitution of other drugs for cocaine, or pre-session treatments on cocaine self-administration were separated by a minimum of 72 hours, and were conducted only if performances met the training criteria. All of the tests were conducted with a mixed order of drugs and doses.

The schedule of food reinforcement was also modified as in the present studies of cocaine self-administration, with five sequential 20-min components, each preceded by a 2-min TO. The first of the five components was EXT (no food available), with an FR 5 schedule of food delivery in effect in the subsequent four components. Subjects were given their daily (~15 g) ration of food (Harlan Rodent Chow) 60 min before sessions, so that their response rates approached those maintained by cocaine.

Once performances were stable across successive sessions, the effects of substitutions for cocaine of saline, dopamine uptake inhibitors, rimcazole analogs, and selective σR antagonists were assessed, with a minimum of 72 hours between treatments. The dopamine uptake inhibitors

studied were WIN 35,428 (0.0032 to 0.1 mg/kg/inj, i.v.), methylphenidate (0.032 to 1.0 mg/kg/inj, i.v.), and nomifensine (0.01 to 0.32 mg/kg/inj, i.v.). Substitutions of rimcazole (0.1 to 3.2 mg/kg/inj, i.v.) and its analogs, SH 3-24 (0.032 to 1.0 mg/kg/inj, i.v.) and SH 3-28 (0.1 to 3.2 mg/kg/inj, i.v.) were compared to those for the σR antagonists, AC927 and NE-100 (0.032 to 1.0 mg/kg/inj, i.v., each). Subsequently, the effects of pre-session i.p. injections of the above drugs on the response rates maintained by cocaine injection or food presentation were assessed. Finally, the effects of combined pre-session i.p. injection of the dopamine uptake inhibitors and the σR antagonists, BD 1008, BD 1047 or BD 1063, on the response rates maintained by cocaine injection or food presentation were also assessed. The dose ranges examined in these experiments are shown in the figures.

Dopamine Transporter Binding Assay: Frozen brains from male Sprague-Dawley rats weighing 200-225 g (Taconic Labs, Germantown, NY) were thawed on ice, the striatum dissected, homogenized in ice cold modified sucrose-phosphate buffer (0.32 M sucrose, 7.74 mM Na_2HPO_4, 2.26 mM NaH_2PO_4, pH adjusted to 7.4) using a Brinkman Polytron (setting 6 for 20 sec), and centrifuged at 50,000 x g for 10 min at 4° C. The resulting pellet was re-suspended in buffer, centrifuged and suspended in buffer again to a concentration of 10 mg/ml original wet weight (OWW).

Ligand binding experiments were conducted in assay tubes containing 0.5 ml sucrose-phosphate buffer. Each tube contained 0.5 nM [^{3}H]WIN 35,428 (specific activity 84 Ci/mmol, PerkinElmer Life Sciences, Waltham, MA) and 1.0 mg striatal tissue, OWW. The reaction was started with the addition of tissue and the tubes were incubated for 120 min on ice. Nonspecific binding was determined using 0.1 mM cocaine HCl (Sigma-Aldrich, St Louis, MO).

σ*R Binding.* Frozen whole guinea-pig brains (minus cerebellum) were thawed on ice, weighed and homogenized (with a glass and Teflon homogenizer) in 10 mM Tris-HCl with 0.32 M sucrose, pH 7.4 (10 ml/g tissue). Guinea-pig brain was used because of the relatively higher density of those receptors in that tissue compared to rat (Tam, 1983). The homogenate was centrifuged at 1000 x g for 10 min at 4° C. The supernatant was collected into a clean centrifuge tube and the remaining pellet was re-suspended by vortex in 10 ml buffer (tissue) and centrifuged again at 50,000 x g for 15 min at 4° C. The resulting pellet was resuspended in experimental buffer to 80 mg/ml, OWW.

Ligand binding experiments were conducted in polypropylene assay tubes containing 0.5 ml of 50 mM Tris-HCl buffer, pH 8.0. For σ_1R binding, each tube contained 3 nM [^{3}H](+)-pentazocine (Perkin Elmer Life Science) and 8.0 mg tissue, OWW. Nonspecific binding was determined using 10 μM haloperidol. For σ_2R binding, each tube contained 3 nM [^{3}H]DTG (Perkin Elmer Life Science), 200 nM (+)-pentazocine, and 8.0 mg tissue, OWW. Nonspecific binding was determined using 100 μM haloperidol. The reaction was started with the addition of tissue and the tubes were incubated for 120 min at room temperature.

Incubations for all binding assays were terminated by rapid filtration through Whatman GF/B filters, presoaked in polyethylenimine, using a Brandel R48 filtering manifold (BRANDEL Instruments Gaithersburg, Maryland). The filters were washed twice with 5 ml ice cold buffer and transferred to scintillation vials. Beckman Ready Safe (3.0 ml) was added and the vials were counted the next day using a Beckman 6000 liquid scintillation counter (Beckman Coulter Instruments, Fullerton, California) at 50% efficiency. Assays were typically conducted in at least three independent experiments, each performed in triplicate.

Drugs. The drugs used in the present study were as follows: (-)-cocaine hydrochloride (Sigma-Aldrich, St. Louis, MO), WIN 35,428 (NIDA, Drug Supply Program), methylphenidate (NIDA), nomifensine (NIDA), rimcazole (Sigma-Aldrich), AC927 (gift from Dr. Andrew Coop), NE-100 (gift from Dr. Tsung-Ping Su), BD 1008 (Tocris, Ballwin, MO), BD 1047 (Tocris), and BD 1063 (Tocris). Rimcazole analogs were synthesized in the Medicinal Chemistry Section, NIDA Intramural Research Program (Husbands et al., 1999). Self-administration of the test drugs was assessed with i.v. delivery of injections, whereas drug pretreatments were administered i.p. All drugs were administered at 5 min before sessions with the exception of BD 1047, which was administered at 15 min before sessions. All drug solutions were prepared fresh daily in 0.9% NaCl, with the exception of SH 3-28 (initially dissolved in 0.16% tartaric acid and with final volumes achieved by adding sterile water). Pretreatment times and doses of drugs used in the present study were chosen based on published (Katz et al., 2003; Hiranita et al., 2010) or preliminary data obtained in this laboratory.

Data analysis. For the radioligand binding assays, the IC_{50} values from the displacement data were computed using a non-linear, least squares regression analysis (GraphPad Prism software, San Diego, CA). Inhibition constants (K_i values) were calculated using the concentration of radioligand employed in the assay, and the historical value for the K_d value of the radioligand determined in this laboratory.

For the behavioral data, response rates were determined by dividing responses by elapsed time in each component, excluding the time outs that followed injections or food presentations. Average values across six subjects (with standard error of the mean) are presented below. To determine if there was a difference in effects of cocaine compared to saline self-administration, a two-way, repeated-measures analysis of variance (ANOVA) was used (factors were component

and substance injected: cocaine or saline). A one-way, repeated measures ANOVA was used to assess the effects of successive components in the substitution for cocaine of the test drugs. A two-way (repeated) measures ANOVA was used to assess the effects of pre-session treatments of the test drugs on cocaine self-administration, and for the comparison of effects of drug pretreatments on responding maintained by cocaine injection (0.32 mg/kg/inj) or food reinforcement. For studies of prior drug treatments on self administration of cocaine, a post-hoc Bonferroni t-test was used for pairwise comparisons. For assessments of the selectivity of drug pretreatments on responding maintained by cocaine injection or food presentation, the effects on responding during the fourth component (in which maximal response rates were maintained by cocaine injection under control conditions) were analyzed by two-way (repeated) measures ANOVA, with a post-hoc Bonferroni t-test used for pairwise comparisons.

RESULTS

Radioligand Binding Assays. Consistent with previous reports (e.g. Izenwasser et al., 1993; Husbands et al., 1999) rimcazole and its *N*-propylphenyl analogs displaced [^{3}H]WIN 35,428 with relatively high affinity. The affinity at the DAT of the diphenylamine, SH 3-24, was comparable to those of the DAT inhibitors, WIN 35,428, nomifensine, and methylphenidate (Fig. 1; Table 1), and higher than that for rimcazole or SH 3-28. In contrast, the K_i values at the DAT for the selective σR antagonists (AC927, BD 1008, BD 1047, NE-100, BD 1063) were at a minimum 10-fold lower than that for SH 3-28, and about 160-fold lower than that for SH 3-24 (Table 1).

Rimcazole had greater affinity than the DAT inhibitors at σ_1 receptors, as labeled with [^{3}H]pentazocine (Fig. 1, Table 1). However, rimcazole was the lowest affinity among recognized σR ligands, and NE-100 was among those with the highest affinity for σ_1Rs, and

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most selective for σ_1 over σ_2 receptors, although that selectivity was only about 50-fold (Table 1).

A previous study using the same methods found the data from [^{3}H]DTG binding, as well as its displacement by several drugs, to be better fit by a two- than a one-site model (Garcés-Ramírez et al., 2010). Similar results were obtained in the present study (Fig. 1). Because the K_i values for known compounds at the higher-affinity site corresponded closely with published literature on σ_2R binding all of the data were fit to a two-site model and the values for the high-affinity site are included in the table proper, with those for the lower-affinity in its footnote. Most of the σR antagonists had higher affinity for the σ_1 than σ_2 receptor, with the most selective being BD 1063. In contrast, rimcazole though having low affinity compared to its analogs, preferentially bound σ_2Rs compared to σ_1Rs. AC927 had a slightly higher affinity at the σ_1R compared to the σ_2R. Finally, K_i values of the DAT inhibitors at either of the σRs were between two and four orders of magnitude greater than their K_i values at the DAT (Fig. 1, Table 1).

Drug Self-Administration Procedures. The average response rates maintained by cocaine were a bell-shaped function of dose, with a maximum of 0.37 ± 0.15 responses/sec when 0.32 mg/kg/inj maintained responding (during the fourth component). This response rate was approximately 10-fold greater than the 0.04 responses/sec occurring during EXT (the first component, Fig. 2A, filled circles above EXT) or those maintained by saline (Fig. 2A, open circles). One-way repeated measures ANOVA indicated a significant effect of cocaine dose on response rate ($p<0.001$). Subsequent replications with cocaine (Fig. 2, panels B-C) showed similar results. Cumulative records of performances show low rates of responding when responses did not produce injections (Fig. 2D, top panel, EXT; first component). When responses produced cocaine injections, brief pauses were followed by a sequence of five responses in rapid sequence until the injection was delivered (Fig. 2, top panel, components 2-5).

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The highest rate of responding was obtained in the fourth component, in which injections of 0.32 mg/kg/inj were available. As was typically observed, the records in Fig. 2 show little or no responses on the inactive lever (perpendicular marks on the lines below the cumulative curve) or during the 2-min TO periods between successive components (lowest line displaced upward). Saline did not maintain rates of responding greater than those obtained in EXT (Fig. 2A; Fig. 2D, second panel); one-way repeated measures ANOVA indicated a non-significant effect of component number on response rate when saline injections were available ($F=0.495$, $p=0.739$).

The dopamine uptake inhibitor WIN 35,428 maintained responding that resembled that maintained by cocaine (Fig. 2B, Fig. 2D, third panel). The highest rate of responding was maintained at a dose of 0.03 mg/kg/inj, with lower response rates at higher and lower doses (Fig. 2B, open circles). The shape of the WIN 35,428 dose-effect curve and the maximal response rates maintained were comparable to those for cocaine; WIN 35,428 was about ten-fold more potent than cocaine (Fig. 2B). A one-way repeated measures ANOVA confirmed statistical significance of effects of WIN 35,428 component on response rates ($F=3.37$, $p=0.025$).

Similar to WIN 35,428, the dopamine uptake inhibitors methylphenidate and nomifensine maintained responding that resembled that maintained by cocaine with regard to maximal response rates and the shape of the dose-effect curves (Fig. 2B). The highest rates of responding maintained by methylphenidate and nomifensine were at doses of 0.32 and 0.1 mg/kg/inj, respectively, with lower response rates at higher and lower doses (Fig. 2B, open triangles up and down, respectively). Methylphenidate was equipotent to cocaine, whereas nomifensine was about three-fold more potent than cocaine (Fig. 2B). Response rates maintained by methylphenidate and nomifensine (F values ≥ 8.48, p values<0.001, one-way repeated measures ANOVA) were significantly affected by dose.

In contrast to the effects of the dopamine uptake inhibitors, no dose of rimcazole (Figs. 2C and fourth panel of Fig 3) nor its analogs, SH 3-24 and SH 3-28, maintained response rates comparable to those maintained by cocaine (Fig. 2C). A one-way repeated measures ANOVA indicated nonsignificant effects of rimcazole ($F=2.19$, $p=0.107$), though the effects of SH 3-24 ($F=4.54$, $p=0.009$) and SH 3-28 ($F_{4,20}=6.76$; $p=0.001$) doses were significant. As can be seen in Fig. 2C, the response rates maintained by either rimcazole analog were only marginally different from those maintained by vehicle, and far below response rates maintained by cocaine or the other DAT inhibitors (Fig. 2B).

Similar to rimcazole and its analogs, no dose of any of the σR antagonists, AC927 and NE-100, maintained response rates comparable to those maintained by cocaine (Fig. 2C). Nonetheless, one-way repeated measures ANOVA indicated significant effects of dose of AC927 ($F_{4,20}=9.46$, $p<0.001$) and NE-100 ($F=71.0$, $p<0.001$). As with rimcazole analogs, none of the significant increases in response rates with these σR antagonists approached those maintained by cocaine, or the other DAT inhibitors (Fig. 2C).

Pretreatment with Rimcazole Analogs. When administered before sessions, rimcazole dose-dependently decreased response rates maintained by cocaine (Fig. 3A). The decreases were expressed as a flattening of the bell-shaped cocaine dose-effect curve. At the highest dose of rimcazole, no dose of cocaine maintained responding at levels appreciably greater than those maintained in EXT (Fig. 3A and Fig. 4, compare first and second panels). Two-way repeated measures ANOVA of the effects of rimcazole on response rates indicated a significant effect of cocaine dose ($F_{4,60}=16.5$; $p<0.001$), pre-session rimcazole treatment ($F_{3,60}=14.2$; $p<0.001$), and the interaction of the two ($F_{12,60}=10.6$; $p<0.001$).

The rimcazole analogs, SH 3-24 and SH 3-28, like the parent compound, dose-

dependently decreased response rates maintained by cocaine (Fig. 3B and C, respectively). At intermediate doses of either compound the maximal effect of cocaine was significantly decreased, and at the highest doses rates of responding were not greater than those obtained in EXT (Fig. 3B and C, compare triangles down to filled circles and to levels maintained in EXT. An ANOVA of the effects on response rates of each of these drugs indicated significant effects of cocaine dose ($F_{4,60}$=8.15 and 6.81, respectively; p values < 0.001), treatment ($F_{3,60}$=4.58 and 5.76, respectively; p values ≤ 0.018), and the interaction of the two ($F_{12,60}$=4.04 and 4.45, respectively; p<0.001).

The effects of rimcazole on responding maintained at the highest response rates by cocaine (at 0.32 mg/kg/inj, fourth component) were compared to effects on responding maintained by food reinforcement at a comparable point in the session (Fig. 3D). Rimcazole produced dose-dependent decreases in rates of responding maintained by cocaine at doses that had no effect on response rates maintained by food reinforcement (Fig. 3D, compare open and filled circles). Two-way repeated measures ANOVA indicated a significant effect of reinforcer ($F_{1,20}$=30.1; p<0.001), a non-significant effect of rimcazole dose ($F_{2,20}$=1.53; p=0.241), though there was a significant interaction of the two ($F_{2,20}$=5.18; p=0.015). Post-hoc analysis indicated that the effects of 32 mg/kg of rimcazole on rates of responding maintained by food and cocaine were significantly different (t=4.60; p<0.001).

SH 3-24 and SH 3-28, also had selective effects on responding maintained by cocaine (Fig. 3E and F, respectively). Little if any effect on response rates maintained by food reinforcement were obtained (open circles) across the range of doses that decreased responding maintained by cocaine. For SH 3-24, ANOVA indicated a significant effect of dose ($F_{2,20}$=6.31; p=0.008), reinforcer ($F_{1,20}$=8.30; p=0.016), and their interaction ($F_{1,20}$=10.4; p<0.001). For SH 3-

28, the ANOVA indicated non-significant effect of dose ($F_{1,20}$=1.30; p=0.294), but a significant effect of reinforcer ($F_{1,20}$=21.8; p<0.001), and a non-significant effect of the interaction ($F_{2,20}$=1.58; p=0.231).

Pretreatment with Selective σR Antagonists or DAT Inhibitors. The selective effects of rimcazole analogs on cocaine self administration prompted studies to assess the relative contributions to their effects of actions at σRs and the DAT using compounds with selectivity for those targets. In contrast to the effects of rimcazole and its analogs, the selective σR antagonists, AC927 and NE-100 (Table 1), generally had no significant effects on the self-administration of cocaine (Fig. 5A and B). Similar results were reported previously with the selective σR antagonists, BD 1008, BD 1047, and BD 1063 (Hiranita et al., 2010). Two-way ANOVA of the effects of AC927 on response rates indicated a significant effect of cocaine dose ($F_{4,60}$=4.66; p=0.008), with non-significant effects of AC927 pretreatment ($F_{3,60}$=0.944; p=0.444), and the interaction the two factors ($F_{12,60}$=1.16; p=0.33). Similarly, the two-way ANOVA of the effect of NE-100 on response rates indicated a significant effect of cocaine dose ($F_{4,60}$=5.23; p=0.005), but effects of neither pre-session treatment ($F_{3,60}$=0.215; p=0.884) nor the interaction of the two ($F_{12,60}$=0.373; p=0.968) were significant.

Pre-session treatment with WIN 35,428 produced a dose-dependent leftward shift in the cocaine self-administration dose-effect curve, without affecting maximum response rate (Fig. 5C). The lowest dose was behaviorally inactive; 0.32 mg/kg of WIN 35,428 shifted the cocaine dose-effect curve approximately 3-fold leftward (Fig. 4, third panel); and 1.0 mg/kg of WIN 35,428 shifted the cocaine dose-effect curve approximately 10-fold leftward, as well as producing an increase in response rates during EXT. The ANOVA results of WIN 35,428 pretreatments indicated significant effects of cocaine dose ($F_{4,60}$=4.47; p=0.010); however, the

effect of the pre-session WIN 35,428 treatment did not achieve statistical significance ($F_{3,60}$=1.22; p=0.335). There was however a significant interaction of cocaine dose and WIN 35,428 pretreatment ($F_{12,60}$=2.99; p=0.002). In addition, post-hoc tests indicated that 1.0 mg/kg of WIN 35,428 increased response rates maintained by 0.03 mg/kg/inj of cocaine (t=3.76; p=0.002), and that 0.32 and 1.0 mg/kg of WIN 35,428 decreased response rates at a cocaine dose of 0.32 mg/kg/inj (t=2.75 and 3.19; p=0.047 and 0.013, respectively).

Pre-session treatments with methylphenidate or nomifensine also produced leftward shifts in the cocaine self-administration dose-effect curve, without affecting maximum response rate (Fig. 5D and E). The lowest doses of both drugs were behaviorally inactive, and doses of 3.2 mg/kg of methylphenidate and 1.0 mg/kg of nomifensine produced approximate 3-fold leftward shifts in the cocaine dose-effect curve, with higher doses producing further shifts, as well as increasing response rates during EXT. The results of ANOVA indicated significant effects of cocaine dose ($F_{4,60}$=7.52; p<0.001; 11.4; p<0.001) and methylphenidate but not nomifensine pretreatment ($F_{3,60}$=4.28; p=0.023; 0.922; p=0.454). With both uptake inhibitors there was a significant interaction of cocaine dose and pretreatment dose ($F_{12,60}$=7.62; p<0.001; 13.4; p<0.001). Additionally, post-hoc tests indicated significant increases in rates of responding maintained with the lowest dose of cocaine by methylphenidate doses of 3.2 and 10 mg/kg (t=3.41 and 4.93; p values≤0.007, respectively).and nomifensine doses of 1.0 and 3.2 mg/kg (t=4.46 and 6.38; p values<0.001, respectively).

Pretreatment with Combinations of Selective σR Antagonists or DAT Inhibitors. Because neither the selective σR antagonists nor the DAT inhibitors reproduced the effects of rimcazole analogs on responding maintained by cocaine, further studies examined whether combinations of those compounds would reproduce the effects of the rimcazole analogs. The

selective σR antagonists used in combination with DAT inhibitors were well studied antagonists synthesized by de Costa and colleagues (de Costa et al., 1992; 1993). These compounds are more widely employed as selective σR antagonists and were used in our previous study with methods identical to those used here (Hiranita et al., 2010). When administered before sessions in combination with 0.1 mg/kg of WIN 35,428, BD 1008 dose-dependently decreased responding maintained by cocaine (Fig. 6A). This effect was obtained despite the fact that the dose of WIN 35,428 as well as all doses of BD 1008 (Hiranita et al., 2010) were behaviorally inactive when administered alone. The ANOVA results (shown in Table 2) indicate significant effects of cocaine dose, pre-session BD 1008 dose in combination with 0.1 mg/kg WIN 35,428, and their interaction. A similar pattern of significant results was obtained with combinations of 0.1 mg/kg WIN 35,428 with BD 1047 (Fig. 6B, Table 2). In contrast, neither the effects of pretreatment with BD 1063 and 0.1 mg/kg WIN 35,428, nor the interaction of that term with cocaine dose were significant (Fig. 6C, Table 2).

In addition, the effects of a higher dose of WIN 35,428 (0.32 mg/kg) were studied with each of the σR antagonists (Fig. 6D-F). That dose of WIN 35,428 in combination with low doses of BD 1008 (0.32, 1.0 mg/kg) shifted the cocaine self-administration dose-effect curve leftward, as did that dose of WIN 35,428 alone. However, higher doses of BD 1008 (3.2, 10.0 mg/kg) resulted in a decrease in maximum effect of cocaine dose/inj (Fig. 6D, see also Fig. 4, bottom panel). The ANOVA indicated a significant effect of cocaine dose, pre-session co-treatment, and their interaction (Table 2). The leftward shift was substantiated by post-hoc tests showing significant effects of 0.32 mg/kg of WIN 35,428 with 1.0 mg/kg dose of BD 1008 at the 0.1 mg/kg/inj cocaine dose (Fig. 6D; Table 2). Similar patterns of effects of combinations of the 0.32 mg/kg dose of WIN 35,428 with BD 1047 and BD 1063 were obtained (Fig. 6E and F), with

similar statistical outcomes (Table 2).

To assess whether the effects of combinations of WIN 35,428 with the various σR antagonists were idiosyncratic to that particular DAT inhibitor, combinations of BD 1008, BD 1047 or BD 1063 with the DAT inhibitors, methylphenidate (Fig. 7A, B and C) or nomifensine (Fig. 7D, E and F), were studied. The doses of the DAT inhibitors chosen for study were those that did not significantly alter the cocaine self-administration dose-effect curve when administered alone (Fig. 5). As with WIN 35,428, doses of either methylphenidate or nomifensine that were behaviorally inactive when administered alone, allowed previously inactive σR antagonists to produce dose-dependent decreases in the maximal effects of cocaine (Fig. 7). ANOVAs of the effects of 1.0 mg/kg of methylphenidate or 0.32 mg/kg nomifensine with BD 1008, BD 1047 or BD 1063 on response rate showed significant effects of cocaine dose, pre-session treatments, and their interactions (Table 2).

The effects of combined pretreatment with dopamine uptake inhibitors and σR antagonists (BD 1008, BD 1047 and BD 1063) on responding maintained at the highest response rates by cocaine (at 0.32 mg/kg/inj, fourth component) were compared to effects on responding maintained by food reinforcement at a comparable point in the session (Fig. 8). As shown in our previous study (Hiranita et al., 2010), the σR antagonists alone had no effect on responding maintained by cocaine at doses that dose-dependently decreased rates of responding maintained by food reinforcement (Fig. 8, left column; results of statistical analyses can be found in Table 3). In contrast, combined treatment with a DAT inhibitor and a σR antagonist reversed the selectivity of the effects obtained with the σR antagonist alone; rates of responding maintained by cocaine were decreased at doses of the σR antagonist combined with the DAT inhibitor that were ineffective on rates of responding maintained by food (Fig. 8B - D). Informal observations

indicated that subjects responding under the schedule of food presentation consumed the pellets delivered during sessions.

Combined treatment with BD 1047 (Fig. 8F - H) or BD 1063 (Fig. 8K - M) with any of the DAT inhibitors also selectively altered responding maintained by cocaine compared to that maintained by food reinforcement. As with BD 1008, the selective effects of BD 1047 (Fig. 8E) or BD 1063 (Fig. 8J) on responding maintained by food reinforcement were reversed by combined treatment with a DAT inhibitor, and the statistical analyses of the effects of each drug combination indicated significant effects of reinforcer in all cases except with WIN 35,428 and BD 1063 (Table 3). In general, the DAT inhibitors attenuated the effects of the σR antagonists on food reinforced behavior and increased their effects on responding maintained by cocaine.

DISCUSSION

In the present study, the σR antagonist, rimcazole, and two of its N-propylphenyl analogs dose-dependently decreased rates of cocaine self-administration. These decreases were obtained at doses that did not affect comparable food-reinforced responding. In contrast, though consistent with previous findings (Martin-Fardon et al., 2007; Hiranita et al., 2010), a number of other σR antagonists did not appreciably alter self-administration of cocaine. In the study by Hiranita et al., pre-session treatments with selective σR antagonists were without effects on cocaine self-administration up to doses three-fold higher than those that reliably antagonized the in vivo effects of σR agonists (Hiranita et al., 2010). Similarly, the cocaine-induced increases in extracellular dopamine levels in the nucleus accumbens shell of rats were not altered by the σR antagonists BD 1008 and BD 1063 (Garcés-Ramírez et al., 2010). Because rimcazole analogs differ from σR antagonists in their additional affinity for the DAT, the present study further assessed the relative contributions of these two sites to the blockade of cocaine self-

administration.

As was shown previously (Barrett et al., 2004; Hiranita et al., 2009; Hiranita et al., 2010), DAT inhibitors (WIN 35,428, methylphenidate, nomifensine) were themselves self-administered, and each shifted the cocaine self-administration dose-effect curve leftward. The decreases in cocaine self-administration with rimcazole analogs show that despite affinity for the DAT, their effects are substantially different from those of the DAT inhibitors. A previous study has also reported the reduced cocaine-like effects of rimcazole analogs (Katz et al., 2003). Acute administrations of rimcazole analogs neither stimulated locomotor activity in mice nor produced cocaine-like discriminative stimulus effects in rats. Further, pretreatments with rimcazole analogs blocked cocaine-stimulated locomotor activity in mice (Katz et al., 2003). Thus rimcazole analogs, though having DAT affinity, generally produce a spectrum of in vivo effects that differ from those of typical DAT inhibitors, such as those presently studied.

Combinations of WIN 35,428 and the σR antagonists, BD 1008 and BD 1047, shifted the cocaine self-administration dose-effect curve downward, suggesting a decrease in the reinforcing effects of cocaine. These effects were similar to those of rimcazole analogs and not specific to WIN 35,428, as similar effects were obtained with methylphenidate and nomifensine. Further and as with the effects of rimcazole analogs, the drug combinations selectively decreased cocaine- compared to food-maintained responding. Interestingly, it appears that DAT inhibition is also necessary to avoid the effects of σR antagonists on food-maintained responding. Thus the present results suggest that the effects of rimcazole analogs on cocaine self-administration result from dual occupancy of the DAT and σRs and that these effects may be selective, suggesting further support for developing these agents as treatments for cocaine abuse (Cao et al., 2003; Katz et al., 2003).

The optimal ratio of σR to DAT affinity cannot be completely addressed without resolution of the relative contributions to the observed effects of σ_1 and σ_2 receptors (see below). From Table 1 it can be seen that the compounds with cocaine-antagonist effects had in vitro σ_1R:DAT affinity ratios between 2 and 10, and in vitro σ_2R:DAT affinity ratios between 2 and 4. As with the selective DAT inhibitors, drugs with high σ_1R:DAT affinity ratios would be expected to produce leftward shifts, suggesting a potentiation of the reinforcing effects of cocaine. However, if the ratio is not excessively weighted towards DAT, the leftward shift would be expressed along with downward shifts, as was obtained with the higher dose of WIN 35,428 in combination with adequate doses of the σR antagonists. The relative dominance of leftward or downward shifts would be expected to vary with the σR:DAT affinity ratio.

It may be noteworthy that higher doses of BD 1008, BD 1047, and possibly BD 1063 were necessary to antagonize cocaine self-administration in combination with WIN 35,428 than with the other DAT inhibitors. Further, the σ_2R affinity of WIN 35,428 is roughly ten-fold higher than that of the other DAT inhibitors, though their σ_1R affinities are similar. It is possible that WIN 35,428 has σR agonist effects, like cocaine (Su and Hayashi, 2001), and that these effects must be overcome by the σR antagonists to produce effects like those of the rimcazole analogs. The lower affinity of nomifensine and methylphenidate at σ_2Rs compared to WIN 35,428 may result in the lower necessary combination doses of the σR antagonists. Further, the σR antagonist with preferential σ_1 affinity (BD 1063) was least effective in selectively decreasing cocaine self-administration in combination with WIN 35,428 than were the other σR antagonists. Differences between BD 1063 and the other σR antagonists were not evident with nomifensine and methylphenidate, DAT inhibitors that had low affinities at both σ_1 and σ_2 receptors. Thus antagonist actions at σ_2Rs may be more involved in the interaction than the σ_1

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subtype. Nevertheless, a clear resolution of the relative contributions of the subtypes of σRs awaits more selective antagonists (Mesangeau et al., 2008).

Previous studies have indicated that cocaine-like dopamine uptake inhibitors bind to the DAT in an outward-facing conformation (Ferrer and Javitch, 1998; Loland et al., 2002) whereas rimcazole analogs bind preferentially to an inward-facing conformation (Loland et al., 2008). Decreases in potency for the inhibition of dopamine uptake in cells transfected with WT DAT compared to a Y335A point-mutated DAT, which assumes an inward-facing conformation, predict whether the compounds will be cocaine-like or atypical in their behavioral effects. Those exhibiting larger decreases in potency are cocaine-like, whereas those that show small changes have reduced cocaine-like effects. WIN 35,428 was among the compounds showing large changes in potency, whereas rimcazole analogs showed much smaller changes in potency (Loland et al., 2008). Thus, DAT conformational equilibrium shifts favored by distinct DAT inhibitors may be related to whether those compounds will produce typical cocaine-like effects. However, WIN 35,428 typically produces effects like those of cocaine and shows a marked decrease in potency in the DAT Y335A mutant compared to wild type (Loland et al., 2008). In addition, both nomifensine and methylphenidate produce effects characteristic of cocaine-like DAT inhibitors, indicating that typical DAT inhibitors can produce effects like those of the atypical DAT inhibitors when administered in combination with σR antagonists. Thus, DAT inhibitors inducing an outward facing DAT conformational equilibrium may produce atypical effects like those of rimcazole when the σR is antagonized.

Previous studies have indicated that endogenous zinc can shift equilibrium of the DAT to an outward-facing conformation (Loland et al., 2002). Further, zinc has been implicated as an endogenous σ_2R ligand (Connor and Chavkin, 1992). Thus any action that mobilizes

endogenous zinc (Frederickson et al., 2005) could alter both the DAT and σRs, though how those actions combine to regulate the effects of cocaine is currently speculative.

Another potential contribution to the interaction between σRs and the DAT is membrane cholesterol. Several studies have documented an involvement of both σ_1 (Hayashi and Su, 2005) and σ_2 (Gebreselassie and Bowen, 2004) receptors in intracellular lipid dynamics. Stimulation of the σ_1R facilitates its translocation to the plasmalemma membrane within which lipid rafts may alter the function of membrane-bound proteins (Hayashi and Su, 2003). Previous studies have demonstrated an influence of membrane lipids on DAT trafficking (Foster et al., 2008) and transport capacity (Adkins et al., 2007). More recently, Hong and Amara (2010) have suggested on the basis of substituted cysteine accessibility methods that a cholesterol-rich membrane environment, as does zinc, shifts the DAT conformational equilibrium towards a state accessible to the extracellular space (outward facing). Again, the potential of an interaction between σRs and the DAT involving membrane cholesterol is currently speculative.

Indirect dopamine agonist actions mediated by the dopamine D_1R have also been recently shown to be influenced by actions at σRs. A recent report by Fu et al. (2010) indicates that the σR agonist, PRE-084, which had no effects of its own, amplified the effects of the D_1R partial agonist, SKF 38393. In addition, Navarro et al. (2010) showed evidence supporting heteromerization of σ and dopamine D_1 receptors, and a potentiated of D_1R-mediated adenylyl cyclase activation by actions at σRs. The reported interactions among σ and dopamine D_1 receptors were also characterized by mutual antagonism of MAPK activation by cocaine acting at σRs and the D_1 agonist, SKF 81297. These studies, including those on zinc and lipids, together suggest several potential mechanisms through which σRs may along with DAT inhibitors modulate the reinforcing effects of cocaine.

Thus, the present study identifies compounds acting on both the DAT and σRs to decrease the self-administration of cocaine. These compounds were not self-administered themselves suggesting low abuse liability of their own. Clinical trials with rimcazole as a potential antipsychotic agent indicated a lack of efficacy (Borison et al., 1991), and an incidence of seizures that halted its further development. While rimcazole therefore may not be an ideal candidate for development as a treatment for cocaine dependence, the present studies suggest that dual inhibition of DAT and σRs can more than adequately blunt the abuse liability resulting from DAT inhibition alone, and further suggest that actions at both sites substantially and selectively block the reinforcing effect of cocaine.

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AUTHORSHIP CONTRIBUTIONS

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Conducted experiments: Hiranita and Kopajtic.

Contributed new reagents or analytic tools: Newman and Cao.

Performed data analysis: Katz and Hiranita.

Wrote or contributed to the writing of the manuscript: Katz, Hiranita, Tanda, Soto, Kohut, Kopajtic, Newman and Cao.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1: Displacement of radioligands for the DAT and σRs by various ligands for the binding sites. Ordinates: Percentage of specific radiotracer bound to membrane preparations as described in Methods. Abscissae: Concentration of each competing compound. The top panel shows binding to the DAT as labeled with [^{3}H]WIN 35,428 the middle panel shows binding to σ_1Rs as labeled with [^{3}H]pentazocine, and the lower panel shows binding to σ_2Rs as labled with [^{3}H]DTG. The curves represent the results of a single experiment with vertical bars representing SEMs from averages of results from three samples. The results were selected from at least three replications as representative of the binding parameters resulting from a global modeling of all of the data.

Figure 2: Substitution of saline, dopamine uptake inhibitors (WIN 35,428, methylphenidate and nomifensine), rimcazole and its analogs (SH 3-24 and SH 3-28), and σR antagonists (AC927 and NE-100) in rats trained to self-administer cocaine. Panel A-C; Ordinates: Responses per sec. Abscissae: Dose of each substituted drug in mg/kg/injection. Each point represents the mean ± SEM (N=6-19). Panel A: Cocaine (filled circles) and saline (open squares). Panel B: Cocaine replication (filled circles), WIN 35,428 (open circles), methylphenidate (open triangles up), nomifensine (open triangles down). Panel C: Cocaine replication (filled circles), rimcazole (open circles), SH 3-24 (open triangles up), SH 3-28 (open triangles down), AC927 (open diamonds) and NE-100 (open squires). Panel D: Representative cumulative records showing patterns of self-administration in real time maintained by intravenous cocaine injection under the fixed-ratio five-response schedule, and those obtained when saline, WIN 35,428, or the σR

antagonist, rimcazole, were substituted for cocaine. Ordinates: cumulative responses. Abscissae: time. The five 20-min self-administration components of each session are indicated by the lower event line displaced down. The preceding 2-min TO periods are indicated by the lower event line displaced up. In the first component each fifth response turned off the LEDs for 20-sec, but did not activate the infusion pump (extinction, EXT), whereas in subsequent components injections were also delivered with each fifth response (diagonal marks on the cumulative record) with doses (in mg/kg/inj) indicated. Vertical marks on the line below the cumulative curve indicate responses on the left (inactive) lever. The cumulative curve reset to the baseline at the end of the 20-min component.

Figure 3: Effects of pre-session treatments with rimcazole and its analogs on responding maintained by cocaine injection or food presentation. Each point represents the mean ± SEM (N=6). Rimcazole and its analogs were administered intraperitoneally at 5 min before sessions. Panel A-C. Effects of pre-session treatments with rimcazole and its analogs on cocaine self-administration. Ordinates: Responses per sec. Abscissae: Cocaine injection dose in mg/kg. Panel A: Effects of rimcazole (3.2, 10 and 32 mg/kg) on cocaine self-administration. Panel B: Effects of SH 3-24 (1.0, 3.2, and 10 mg/kg) on cocaine self-administration. Panel C: Effects of SH 3-28 (3.2, 10 and 32 mg/kg) on cocaine self-administration. Panel D-E. Effects of pre-session treatments with rimcazole and its analogs on responding maintained by cocaine injection (0.32 mg/kg/inj) or food presentation (fourth component, each). Ordinates: Response rates as percentage of control response rates (sessions prior to drug tests), which averaged 0.40 (±0.13), and 0.91 (±0.10) responses per sec, respectively, for cocaine- and food-maintained responding. Abscissae: mg/kg of test compounds administered i.p., log scale. Closed and open circles

indicate behavior maintained by cocaine (0.32 mg/kg/inj, i.v.) and food presentation, respectively.

Figure 4: Representative cumulative records showing patterns of self-administration maintained by intravenous cocaine injection under the fixed-ratio five-response schedule with the particular treatments before sessions as indicated in each panel of the figure. All details are as in Figure 1D.

Figure 5: Effects of pre-session treatments with σR antagonists or dopamine uptake inhibitors on cocaine self-administration in rats trained to self-administer cocaine. Ordinates: Responses per sec. Abscissae: Cocaine injection dose in mg/kg. Each point represents the mean ± SEM (N=6). AC927 was administered intraperitoneally at 15 min before sessions, whereas the other drugs at 5 min before sessions. Panel A: Effects of the σR antagonist AC927 (1.0, 3.2 and 10 mg/kg) on cocaine self-administration. Panel B: Effects of NE-100 (1.0, 3.2 and 10 mg/kg) on cocaine self-administration. Panel C: Effects of WIN 35,428 (0.1, 0.32, and 1.0 mg/kg) on cocaine self-administration. Panel D: Effects of methylphenidate (1.0, 3.2, and 10 mg/kg) on cocaine self-administration. Panel E: Effects of nomifensine (0.32, 1.0, and 3.2 mg/kg) on cocaine self-administration.

Figure 6: Effects of pre-session treatments with WIN 35,428 combined with σR antagonists on cocaine self-administration. Ordinates: Responses per sec. Abscissae: Cocaine injection dose in mg/kg. Each point represents the mean ± SEM (N=6). WIN 35,428, BD1008, BD1047, and BD1063 were administered intraperitoneally at 5, 5, 15, 5 min before sessions, respectively. Panel A: Effects of WIN 35,428 (0.1 mg/kg) with BD 1008 (3.2 and 10 mg/kg) on cocaine self-

administration. Panel B: Effects of WIN 35,428 (0.1 mg/kg) with BD 1047 (3.2 and 10 mg/kg) on cocaine self-administration. Panel C: Effects of WIN 35,428 (0.1 mg/kg) with BD 1063 (3.2 and 10 mg/kg) on cocaine self-administration. Panel D: Effects of WIN 35,428 (0.32 mg/kg) with BD 1008 (0.32, 1.0, 3.2 and 10 mg/kg) on cocaine self-administration. Panel E: Effects of WIN 35,428 (0.32 mg/kg) with BD 1047 (0.32, 1.0, 3.2 and 10 mg/kg) on cocaine self-administration. Panel F: Effects of WIN 35,428 (0.32 mg/kg) with BD 1063 (0.32, 1.0, 3.2 and 10 mg/kg) on cocaine self-administration.

Figure 7: Effects of pre-session treatments with methylphenidate (1.0 mg/kg) or nomifensine (0.32 mg/kg) combined with σR antagonists on cocaine self-administration. Ordinates: Responses per sec. Abscissae: Cocaine injection dose in mg/kg. Each point represents the mean ± SEM (N=6). Methylphenidate, BD1008, BD1047, and BD1063 were administered intraperitoneally at 5, 5, 15, 5 min before sessions, respectively. Panel A: Effects of methylphenidate (1.0 mg/kg) with BD 1008 (1.0, 3.2 and 10 mg/kg) on cocaine self-administration. Panel B: Effects of methylphenidate with BD 1047 (1.0, 3.2 and 10 mg/kg) on cocaine self-administration. Panel C: Effects of methylphenidate with BD 1063 (1.0, 3.2 and 10 mg/kg) on cocaine self-administration. Panel D: Effects of nomifensine with BD 1008 (1.0, 3.2 and 10 mg/kg) on cocaine self-administration. Panel E: Effects of nomifensine with BD 1047 (1.0, 3.2 and 10 mg/kg) on cocaine self-administration. Panel F: Effects of nomifensine with BD 1063 (1.0, 3.2 and 10 mg/kg) on cocaine self-administration.

Figure 8: Effects of pre-session treatments with dopamine uptake inhibitors combined with σR antagonists on responding maintained by cocaine injection or food presentation. Ordinates:

Response rates as percentage of control response rates (sessions prior to drug tests). Abscissae: mg/kg of σR antagonists administered i.p. in combination with designated dopamine uptake inhibitors, log scale. Dopamine uptake inhibitors and σR antagonists were administered i.p. 5 min before sessions except BD 1047 (15 min). Responding was from the 4th 20-min component of the session (see Methods). All other details are as in Figure 2D-F.

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Table 1: Inhibition by various compounds of specific binding to the DAT, σ_1, or σ_2 receptors. The values listed are K_i values with 95% confidence limits in parentheses, with the exception of the value for WIN 35,428 at the DAT, which is a K_d value obtained from a homologous competition study. See Methods section for details of the assay procedures and derivation of K_i values.

Compound	**DAT K_i Value ± SEM (nM) or 95% CLs**	**σ_1 K_i Value ± SEM (nM) or 95% CLs**	**σ_2 K_i Value ± SEM (nM) or 95% CLs**
WIN 35,428	5.24* (4.92 – 5.57)	5,700 (4,060 – 8,020)	4,160 (3,120 – 5,550)
SH 3-24	12.2 (10.8 – 13.8)	22.9 (18.5 – 28.2)	20.0 (15.7 – 25.6)
Nomifensine	21.0 (18.9 – 23.3)	8,240 (5,360 – 12,700)	65,200 (54,300 – 78,300)
Methylphenidate	65.8 (61.2 – 70.8)	6,780 (4,520 – 10,200)	37,400 (21,200 – 66,100)
Cocaine[†]	76.6 (72.6 - 80.5)	5,190 (3,800 – 7,060)	19,300 (16,000 - 23,300)
Rimcazole	96.6 (77.3 – 121)	883 (661 – 1,180)	238 (171 – 329)
SH 3-28	188 (166 – 213)	19.0 (15.3 – 23.6)	47.2 (40.4 – 55.2)

AC927	1,930 (1,610 – 2,320)	53.1 (45.6 – 61.8)	78.9 (48.2 – 129)
BD 1008†	2,510 (2,250 – 2,790)	2.13 (1.77 – 2.56)	16.6 (13.0 - 21.1)
BD 1047†	3,220 (2,820 – 3,670)	3.13 (2.68 – 3.65)	47.5 (36.7 - 61.4)
NE-100	3,590 (3,210 – 4,000)	2.48 (2.13 – 2.88)	121 (91.9 – 159)
BD 1063†	8,020 (7,100 – 9,060)	8.81 (7.15 – 10.9)	625 (447 - 877)

*The value for affinity of WIN 35,428 at the DAT is a K_d value obtained from a homologous competition study.

†The values for these compounds are those reported in the literature by Garcés-Ramírez et al., 2010 using methods identical to those in the present study. For the [^{3}H]DTG assay, the data typically modeled better for two than one binding site, and the K_i values for the higher affinity site are displayed in the table. The DTG high-affinity site is the site recognized as the σ_2 receptor, whereas the low affinity site is currently not identified. Values for the low affinity DTG site and their 95% confidence limits in nM were as follows: SH 3-24:12,700 (1,300 – 124,000); rimcazole: 25,900 (3,620 – 185,000); AC927: 55,200 (6,860 - 444,000), BD 1008: 20,500 (9,640 – 43,500); BD 1047: 55,300 (25,000 – 122,000); BD 1063: 53,700 (16,500 - 174,000).

Table 2: Results of statistical analysis of the effects of drug combinations on cocaine self-administration.

Treatment	**Cocaine Dose**	**Pretreatment**	**Interaction**	**Post-hoc test at 0.1 mg/kg/inj Cocaine**	**Post-hoc test at 0.32 mg/kg/inj Cocaine**
0.1 mg/kg WIN 35,428 & BD 1008	$F_{4,40}$=6.38; p=0.002	$F_{2,40}$=4.51; p=0.040	$F_{8,40}$=3.57; p=0.003	N.S.	10 mg/kg BD 1008, t=5.84; p<0.001
0.1 mg/kg WIN 35,428 & BD 1047	$F_{4,40}$=21.8; p<0.001	$F_{2,40}$=1.22; p=0.337	$F_{8,40}$=1.94; p=0.081	N.S.	N.S.
0.1 mg/kg WIN 35,428 & BD 1063	$F_{4,40}$=4.15; p=0.013	$F_{2,40}$=0.601; p=0.567	$F_{8,40}$=0.63; p=0.748	N.S.	N.S.
0.3 mg/kg WIN 35,428 & BD 1008	$F_{4,80}$=6.91; p=0.001	$F_{4,80}$=5.68; p=0.003	$F_{16,80}$=4.62; p<0.001	1.0 mg/kg BD 1008, t=3.00; p=0.034	0.32 - 10 mg/kg BD 1008, t=5.50, 5.59, 5.62 and 6.12, respectively; p values <0.001
0.3 mg/kg WIN 35,428 & BD 1047	$F_{4,80}$=11.8; p<0.001	$F_{4,80}$=1.65; p=0.201	$F_{16,80}$=2.97; p<0.001	0.32 mg/kg BD 1047, t=4.03; p≤0.002 1.0 mg/kg BD 1047, t=3.90, p≤0.002	N.S.
0.3 mg/kg WIN 35,428	$F_{4,80}$=14.2;	$F_{4,80}$=3.33;	$F_{16,80}$=4.54;	0.32 mg/kg BD 1063, t=6.09, p≤0.009	N.S.

& BD 1063	p<0.001	p=0.030	p<0.001	1.0 mg/kg BD 1063, t=3.42, p≤0.009	
1.0 mg/kg MPD & BD 1008	$F_{4,60}$=8.54; p<0.001	$F_{3,60}$=8.06; p=0.002	$F_{12,60}$=7.27; p<0.001	3.2 mg/kg BD 1008, t=3.15; p≤0.016 10 mg/kg BD 1008, t=4.37, p≤0.016	3.2 mg/kg BD 1008, t=4.54; p<0.001 10 mg/kg BD 1008, t=6.62; p<0.001
1.0 mg/kg MPD & BD 1047	$F_{4,60}$=8.41; p<0.001	$F_{3,60}$=8.98; p=0.001	$F_{12,60}$=7.10; p<0.001	3.2 mg/kg BD 1047, t=4.44, p<0.001 10 mg/kg BD 1047, t= 4.31, p<0.001	3.2 mg/kg BD 1047, t=5.99; p<0.001 10 mg/kg BD 1047, t=7.02; p<0.001
1.0 mg/kg MPD & BD 1063	$F_{4,60}$=8.99; p<0.001	$F_{3,60}$=8.77; p=0.001	$F_{12,60}$=7.56; p<0.001	3.2 mg/kg BD 1063, t=4.24; p<0.001 10 mg/kg BD 1063, t=4.37, p<0.001	3.2 mg/kg BD 1063, t=5.64; p<0.001 10 mg/kg BD 1063, t= 6.99; p<0.001
0.32 mg/kg Nomifensine & BD 1008	$F_{4,60}$=14.7; p<0.001	$F_{3,60}$=13.7; p<0.001	$F_{12,60}$=12.8; p<0.001	3.2 mg/kg BD 1008, t=6.11, p<0.001 10 mg/kg BD 1008, t=5.91, p<0.001	3.2 mg/kg BD 1008, t=7.35; p<0.001 10 mg/kg BD 1008, t=9.00; p<0.001
0.32 mg/kg Nomifensine & BD 1047	$F_{4,60}$=13.3; p<0.001	$F_{3,60}$=12.9; p=0.001	$F_{12,60}$=11.8; p<0.001	3.2 mg/kg BD 1047, t=4.63; p<0.001 10 mg/kg BD 1047, t=5.77, p<0.001	3.2 mg/kg BD 1047, t=5.26; p<0.001 10 mg/kg BD 1047, t=8.26; p<0.001
0.32 mg/kg Nomifensine & BD 1063	$F_{4,60}$=16.3; p<0.001	$F_{3,60}$=14.9; p=0.001	$F_{12,60}$=13.3; p<0.001	3.2 mg/kg BD 1063, t=6.19, p<0.001 10 mg/kg BD 1063, t=6.30, p<0.001	3.2 mg/kg BD 1063, t=7.31; p<0.001 10 mg/kg BD 1063, t=9.22; p<0.001

Table 3: Results of statistical analysis of the effects of drug combinations on cocaine self-administration compared to food-maintained behavior.

Treatment	**Reinforcer**	**Dose**	**Interaction**	**Post-hoc test of Reinforcer**
BD 1008 & Saline	$F_{1,15}$=21.5; p<0.001	$F_{2,15}$=5.88; p=0.013	$F_{2,15}$=0.675; p=0.524	10 mg/kg BD 1008, t=2.67, p=0.012 32 mg/kg BD 1008, t=3.04, p=0.005
BD 1047 & Saline	$F_{1,15}$=99.1; p<0.001	$F_{2,15}$=0.485; p=0.625	$F_{2,15}$=4.68; p=0.026	10 mg/kg BD 1047, t=5.78; p<0.001 32 mg/kg BD 1047, t=6.38, p<0.001
BD 1063 & Saline	$F_{1,20}$=5.01; p=0.049	$F_{2,20}$=4.72; p=0.021	$F_{2,20}$=2.83; p=0.083	32 mg/kg BD 1063, t=2.98, p=0.006
0.1 mg/kg WIN 35,428 & BD 1008	$F_{1,10}$=2.57; p=0.140	$F_{1,10}$=3.65; p=0.085	$F_{1,10}$=0.598; p=0.457	N.S.
0.3 mg/kg WIN 35,428 & BD 1008	$F_{1,30}$=16.2; p=0.002	$F_{3,30}$=0.944; p=0.432	$F_{3,30}$=0.640; p=0.595	0.32 mg/kg BD 1008, t=2.52; p=0.018 1.0 mg/kg BD 1008, t=2.89, p=0.008 3.2 mg/kg BD 1008, t=3.91, p<0.001

				10 mg/kg BD 1008, t=2.75, p=0.011
1.0 mg/kg Methylphenidate & BD 1008	$F_{1,20}$=10.9; p=0.008	$F_{2,20}$=4.01; p=0.034	$F_{2,20}$=9.19; p=0.001	3.2 mg/kg BD 1008, t=2.60; p=0.014 10 mg/kg BD 1008, t=4.73, p<0.001
0.32 mg/kg Nomifensine & BD 1008	$F_{1,20}$=24.9; p<0.001	$F_{2,20}$=3.25; p=0.060	$F_{2,20}$=19.7; p<0.001	3.2 mg/kg BD 1008, t=3.92, p<0.001 10 mg/kg BD 1008, t=7.27, p<0.001
0.1 mg/kg WIN 35,428 & BD 1047	$F_{1,10}$=7.95; p=0.018	$F_{1,10}$=7.55; p=0.021	$F_{1,10}$=60.0; p<0.001	N.S.
0.3 mg/kg WIN 35,428 & BD 1047	$F_{1,30}$=158; p<0.001	$F_{3,30}$=3.16; p=0.039	$F_{3,30}$=0.717; p=0.550	0.32 mg/kg BD 1047, t=6.02; p<0.001 1.0 mg/kg BD 1047, t=5.83, p<0.001 3.2 mg/kg BD 1047, t=4.20; p<0.001 10 mg/kg BD 1047, t=5.98, p<0.001
1.0 mg/kg Methylphenidate & BD 1047	$F_{1,20}$=17.2; p=0.002	$F_{2,20}$=34.3; p<0.001	$F_{2,20}$=0.166; p=0.848	1.0 mg/kg BD 1047, t=3.30, p=0.003 3.2 mg/kg BD 1047, t=2.94, p=0.008 10 mg/kg BD 1047, t=3.55, p=0.002
0.32 mg/kg Nomifensine	$F_{1,20}$=9.25;	$F_{2,20}$=14.02;	$F_{2,20}$=8.50;	3.2 mg/kg BD 1047, t=3.93; p<0.001

& BD 1047	p=0.012	p<0.001	p=0.002	10 mg/kg BD 1047, t=3.39, p=0.003
0.1 mg/kg WIN 35,428 & BD 1063	$F_{1,10}$=0.284 ; p=0.606	$F_{1,10}$=1.71; p=0.220	$F_{1,10}$=0.375; p=0.554	N.S.
0.3 mg/kg WIN 35,428 & BD 1063	$F_{1,30}$=85.9; p<0.001	$F_{3,30}$=0.690; p=0.565	$F_{3,30}$=1.27; p=0.303	0.32 mg/kg BD 1063, t=2.42, p=0.021 1.0 mg/kg BD 1063, t=3.97, p<0.001 3.2 mg/kg BD 1063, t=2.68, p=0.011 10 mg/kg BD 1063, t=5.00, p<0.001
1.0 mg/kg Methylphenidate & BD 1063	$F_{1,20}$=19.5; p=0.001	$F_{2,20}$=0.377; p=0.690	$F_{2,20}$=3.91; p=0.037	3.2 mg/kg BD 1063, t=3.37; p=0.002 10 mg/kg BD 1063, t=3.72, p<0.001
0.32 mg/kg Nomifensine & BD 1063	$F_{1,20}$=70.9; p<0.001	$F_{2,20}$=1.95; p=0.168	$F_{2,20}$=13.7; p<0.001	3.2 mg/kg BD 1063, t=4.92, p<0.001 10 mg/kg BD 1063, t=7.99, p<0.001

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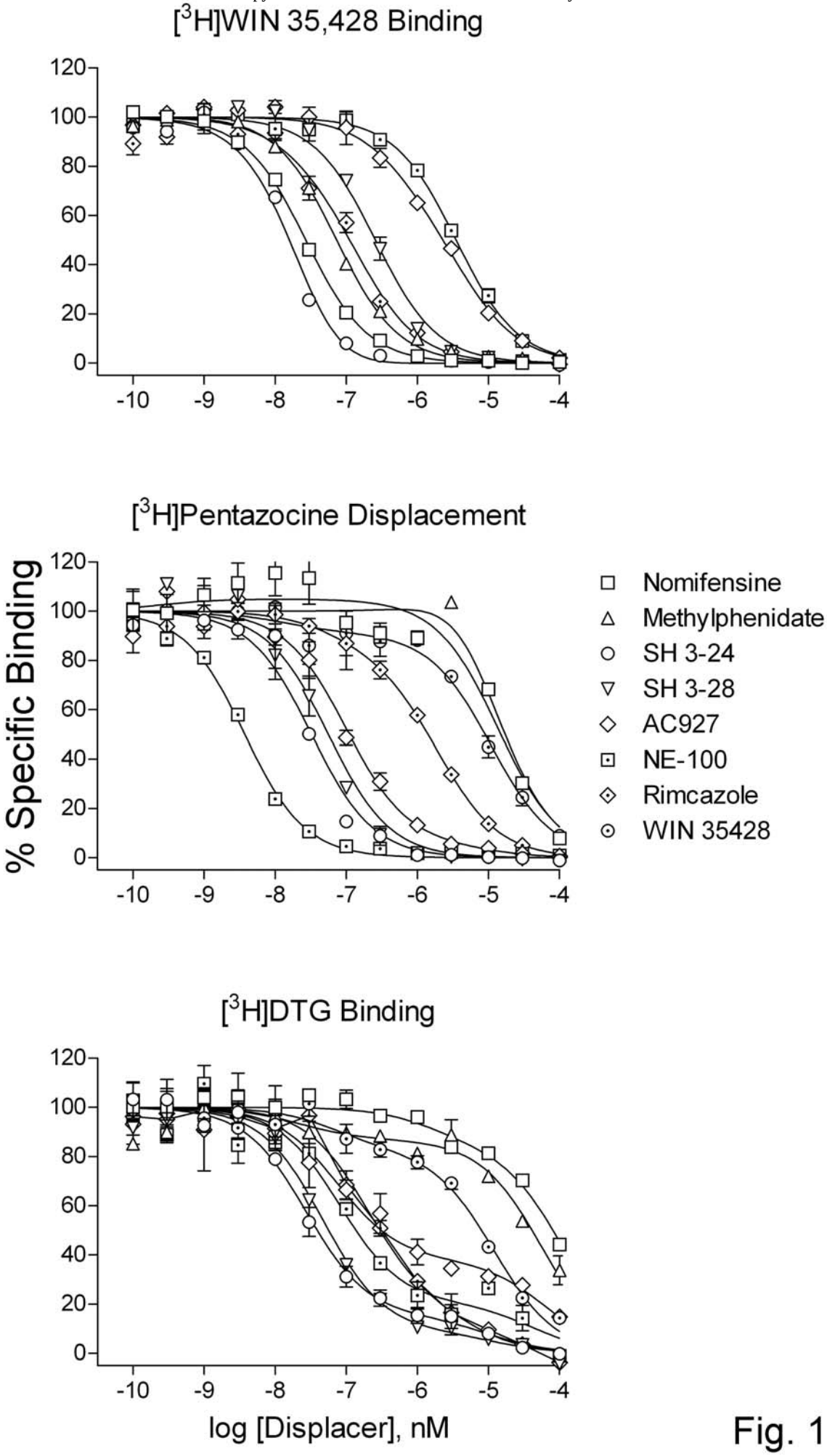

Fig. 1

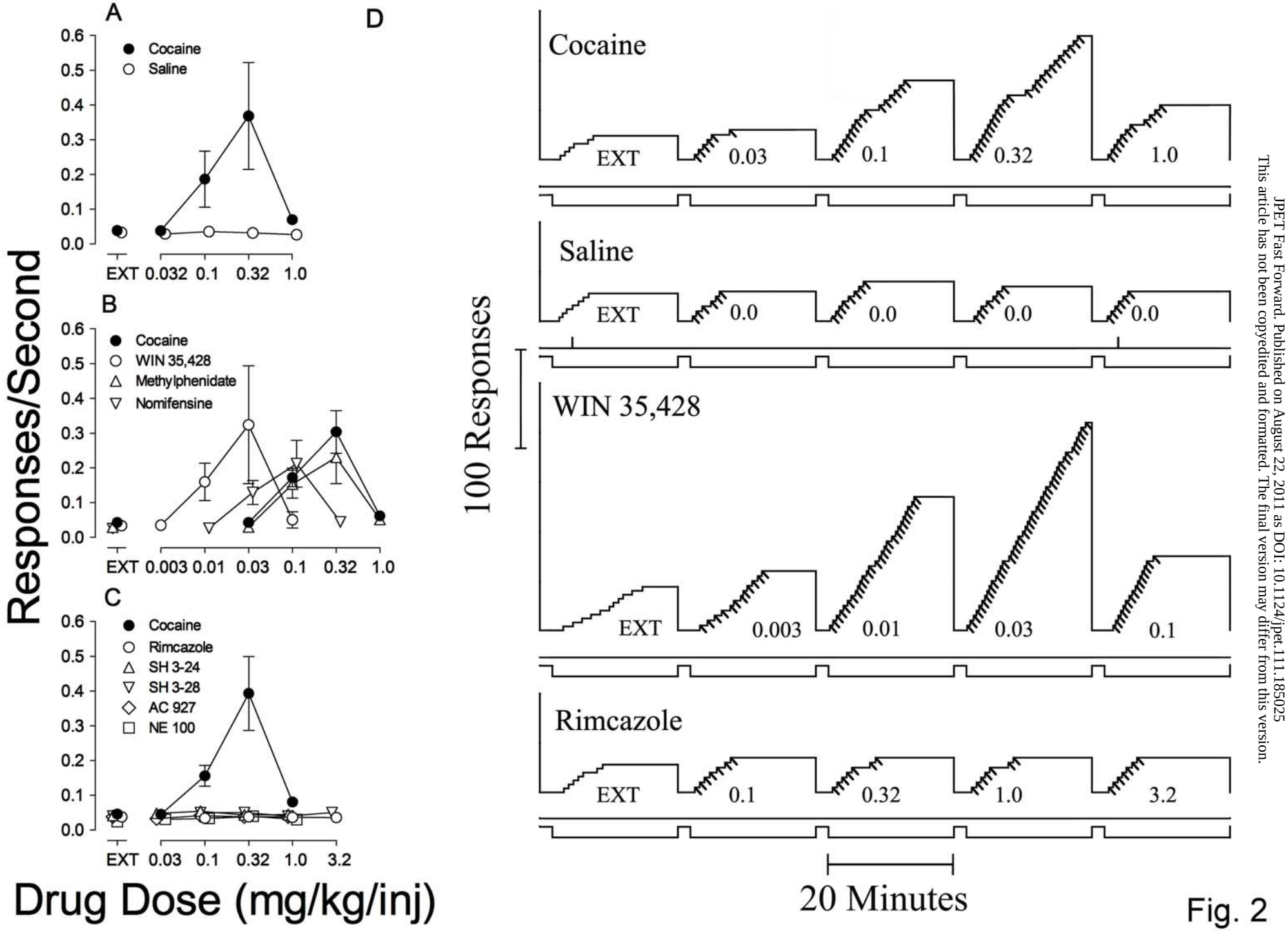

Fig. 2

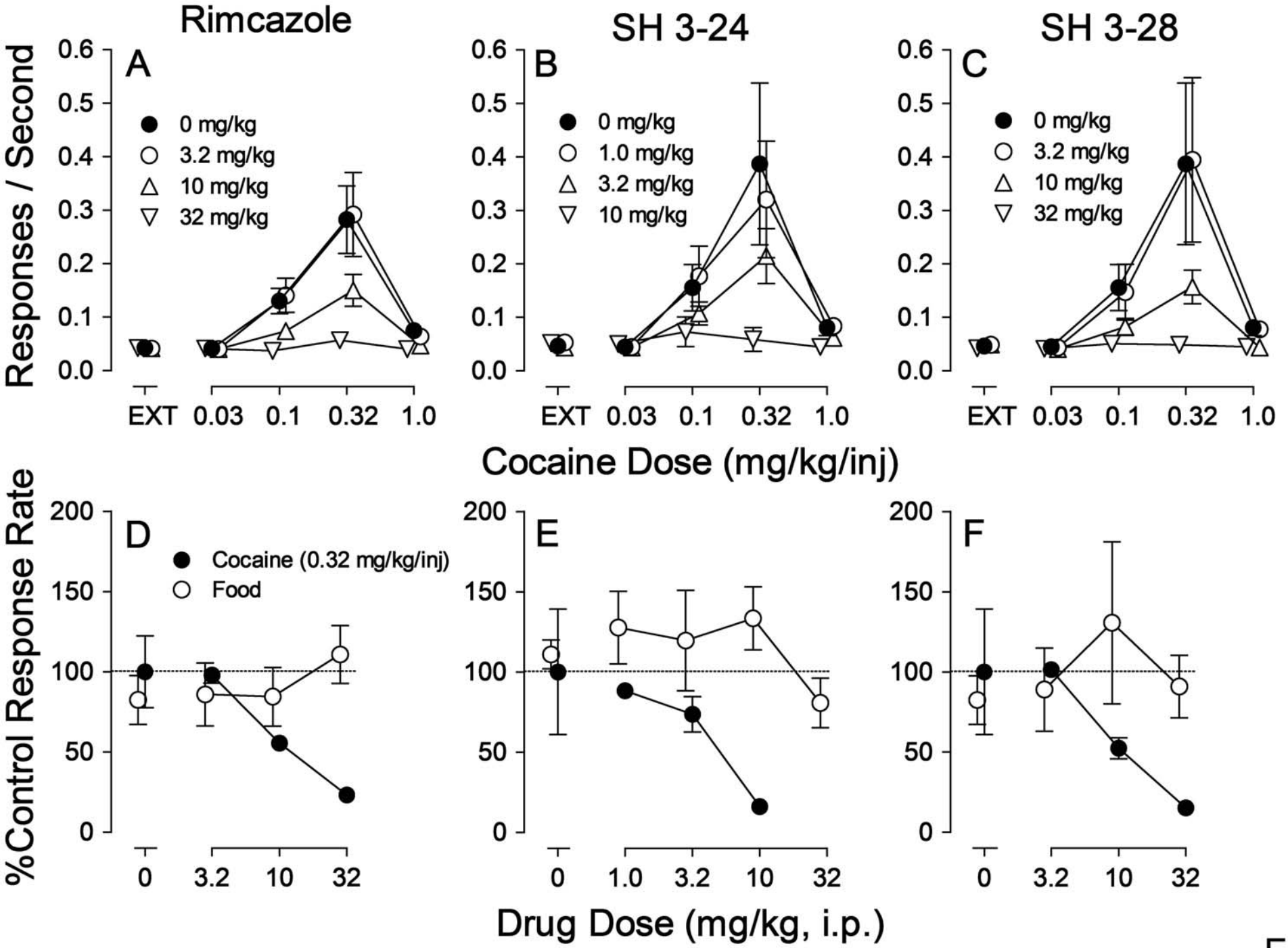

Fig. 3

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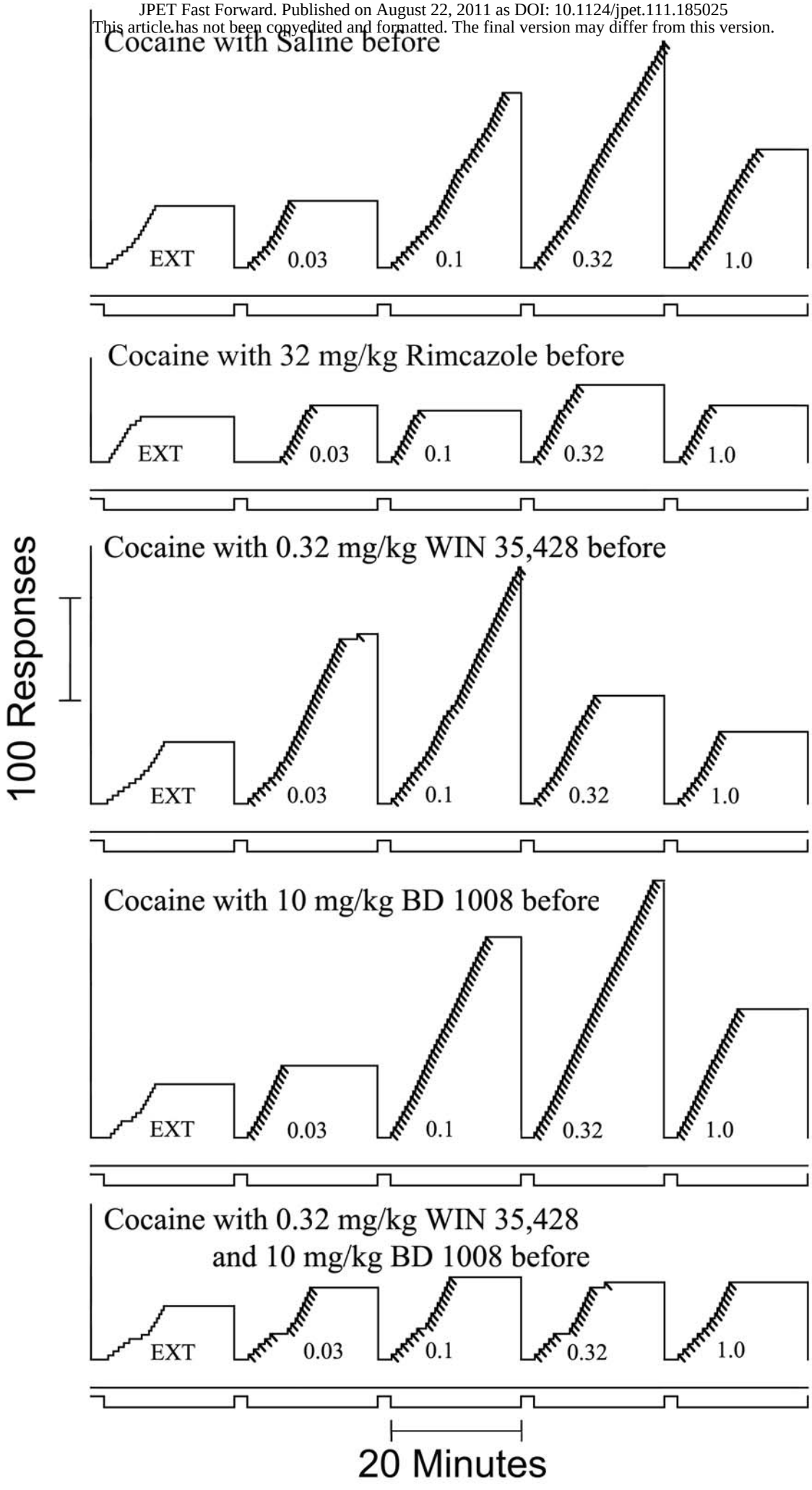

Fig. 4

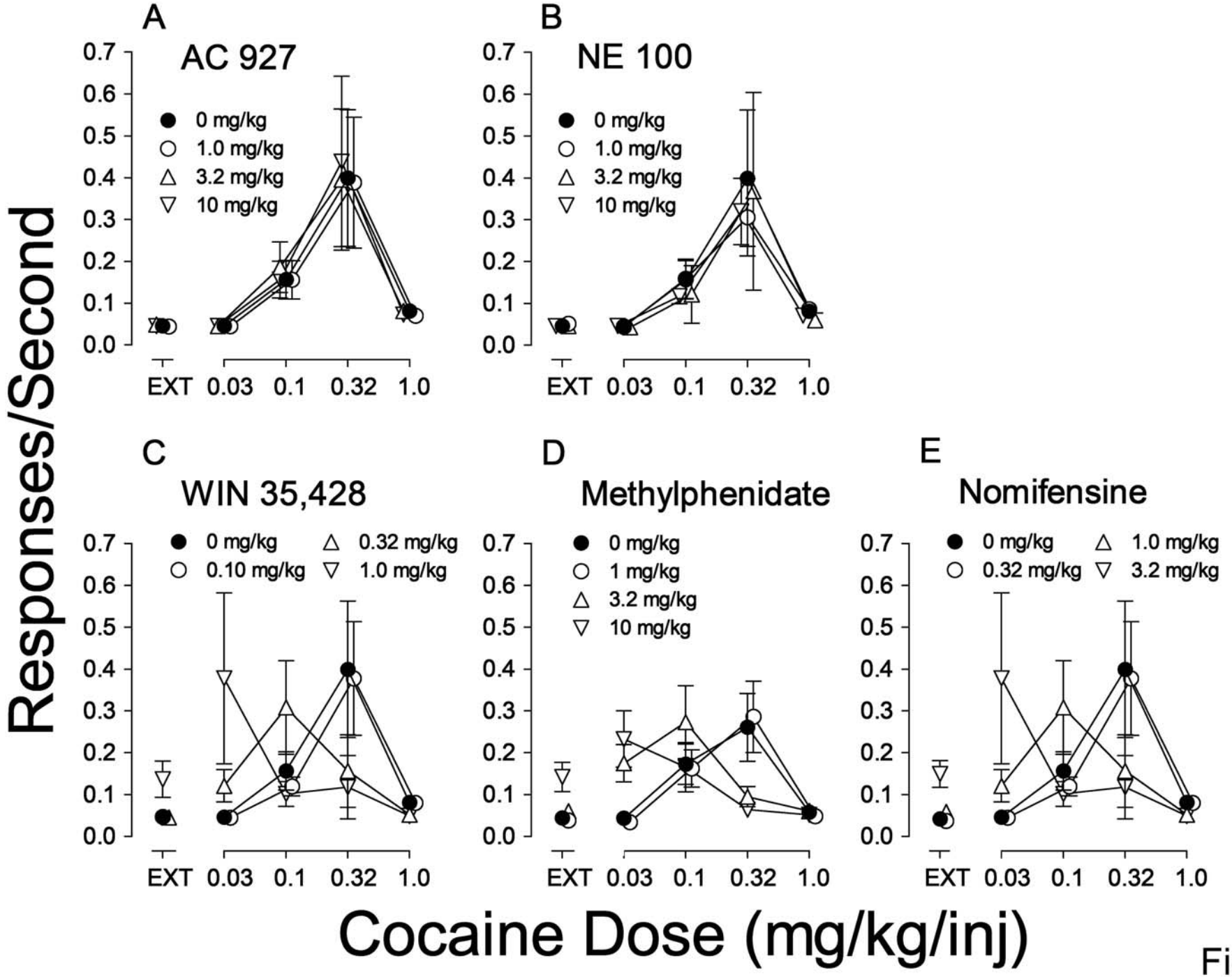

Fig. 5

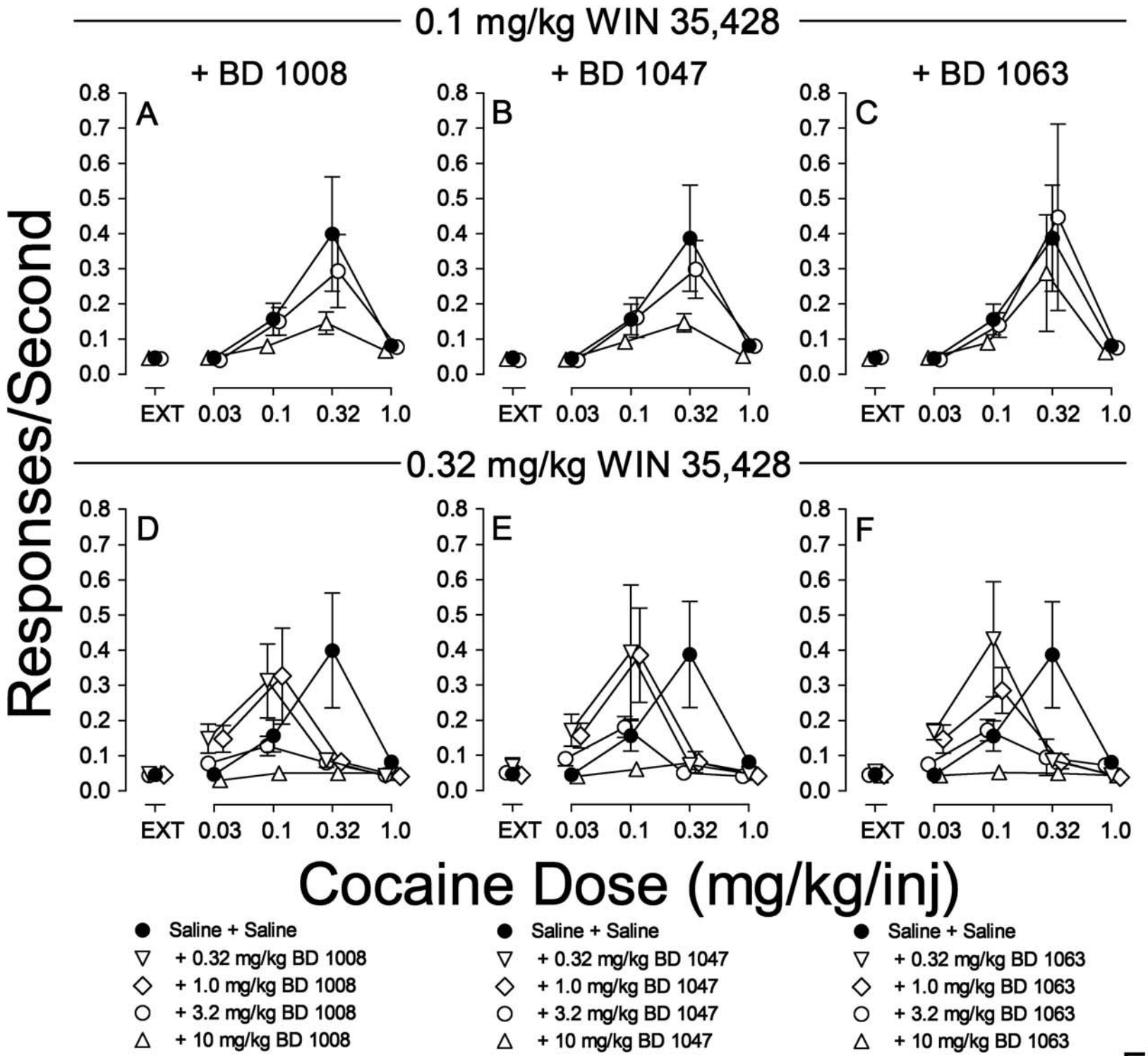

Fig. 6

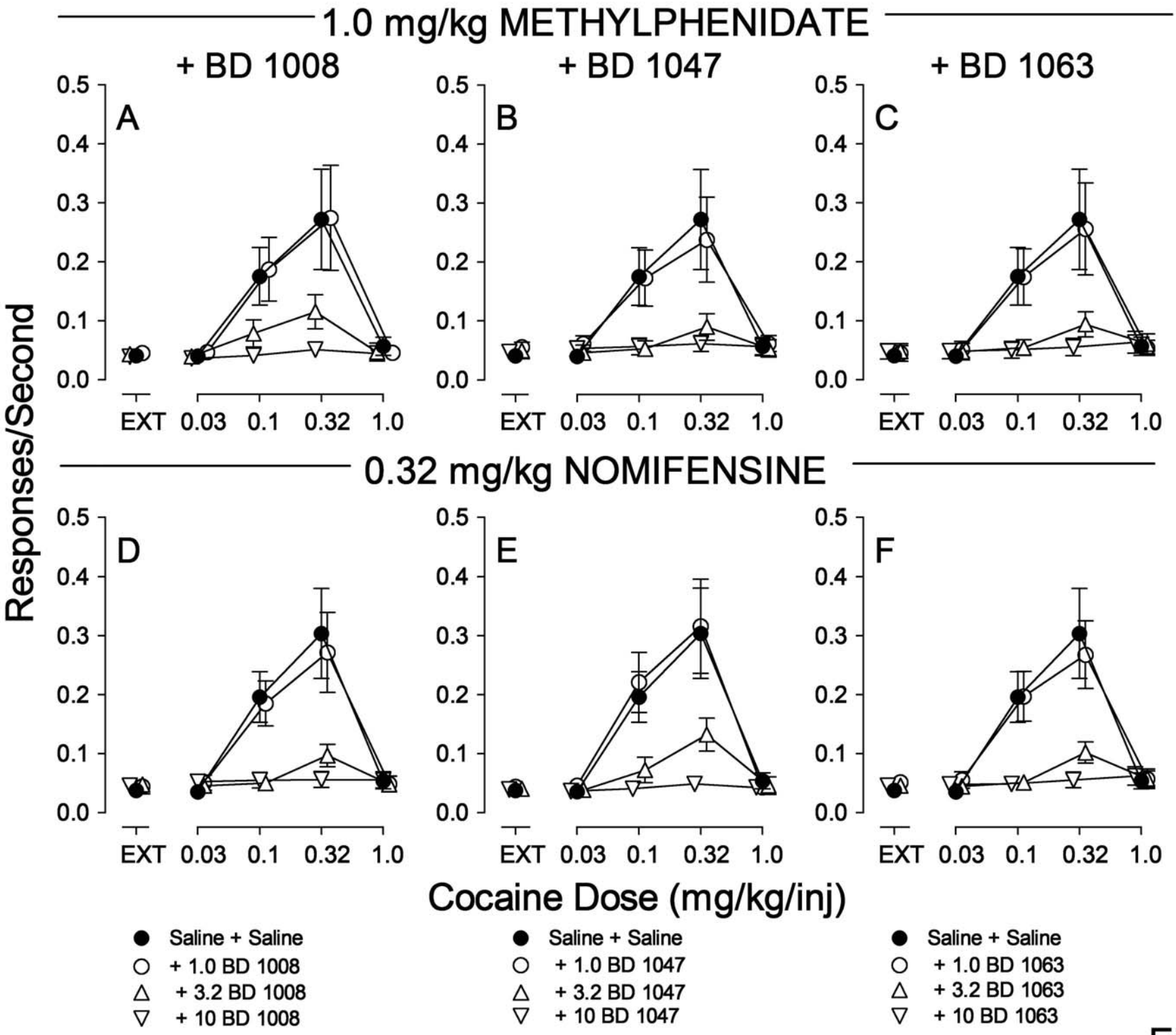

Fig. 7

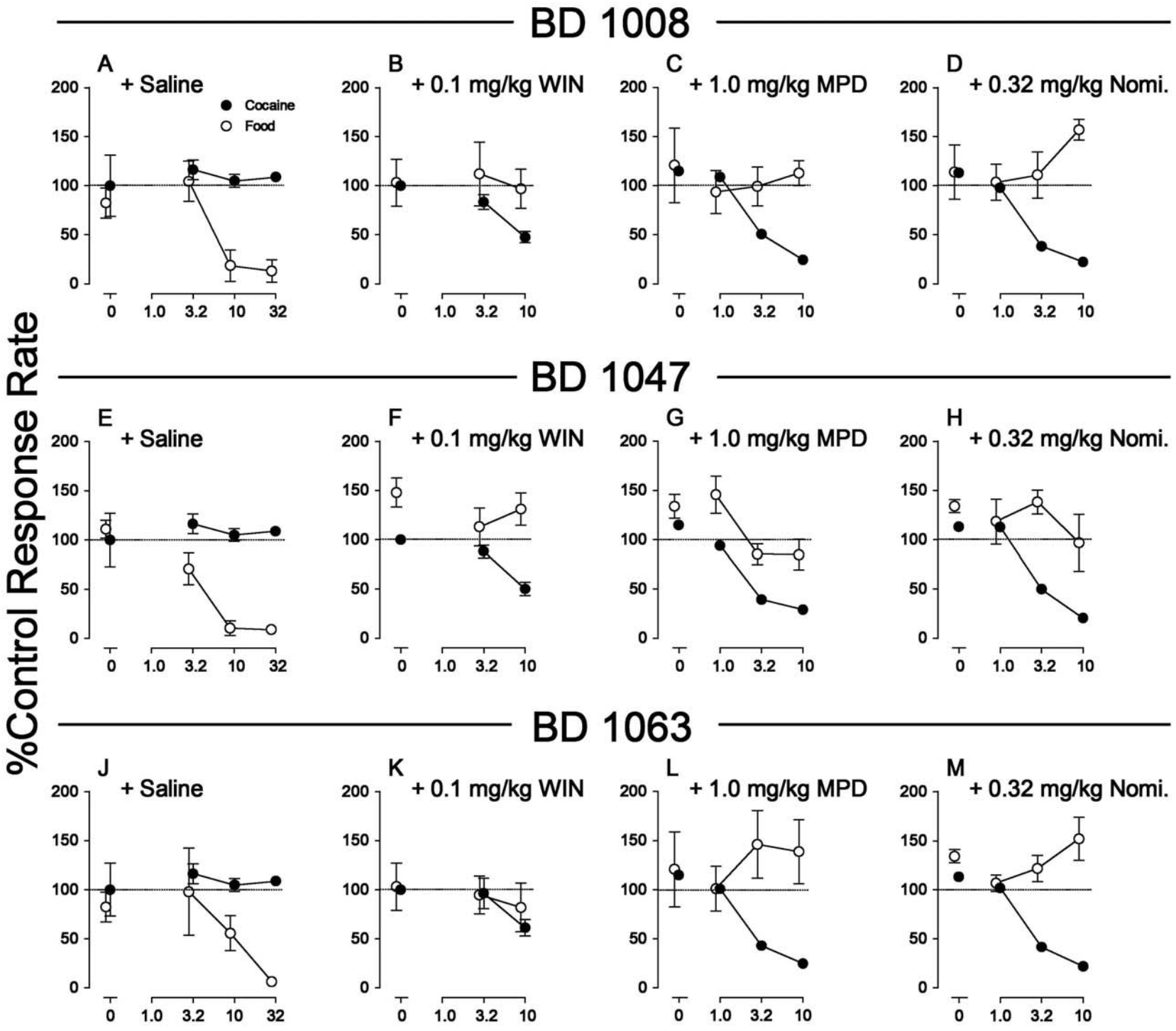

Fig. 8