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The Novel Dopamine D_3 Receptor Antagonist NGB 2904 Inhibits Cocaine's Rewarding Effects and Cocaine-Induced Reinstatement of Drug-Seeking Behavior in Rats

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Accumulating evidence indicates that dopamine (DA) D_3 receptor antagonists appear highly promising in attenuating cocaine reward and relapse in preclinical models of addiction. In the present study, we investigated the effects of the novel D_3-selective antagonist NGB 2904 (*N*-(4-[4-{2,3-dichlorophenyl}-1-piperazinyl]butyl)-3-fluorenylcarboxamide) on cocaine self-administration, cocaine-enhanced brain stimulation reward (BSR), and cocaine-triggered reinstatement of drug-seeking behavior in male Long–Evans rats. We found that: (1) acute intraperitoneal (i.p.) administration of NGB 2904 (0.1–10 mg/kg) failed to alter cocaine self-administration (0.5 mg/kg/infusion) under fixed-ratio 2 (FR2) reinforcement, but 1 or 5 mg/kg NGB 2904 significantly lowered the break-point for cocaine self-administration under progressive-ratio (PR) reinforcement; (2) cocaine (1, 2, and 10 mg/kg) significantly enhanced electrical BSR (decreased brain reward thresholds), while NGB 2904 significantly inhibited the enhancement of BSR elicited by 2 mg/kg, but not 10 mg/kg of cocaine; (3) NGB 2904 alone neither maintained self-administration behavior nor altered brain reward thresholds; and (4) NGB 2904 significantly inhibited cocaine-triggered reinstatement of extinguished drug-seeking behavior, but not sucrose-plus-sucrose-cue-triggered reinstatement of sucrose-seeking behavior. Overall, these data show that the novel D_3-selective antagonist NGB 2904 attenuates cocaine's rewarding effects as assessed by PR self-administration, BSR, and cocaine-triggered reinstatement of cocaine-seeking behavior. Owing to these properties and to its lack of rewarding effects (as assessed by BSR and by substitution during drug self-administration), NGB 2904 merits further investigation as a potential agent for treatment of cocaine addiction.
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INTRODUCTION

Dopamine (DA) D_3 receptors have recently drawn attention as a potential target for medication development for drug addiction, in part because of their unique regional distribution in the mesolimbic DA system (Murray *et al*, 1994; Levant, 1997; Stanwood *et al*, 2000). Virtually all addictive drugs activate the mesolimbic DA system (see for a review Gardner, 2005), and this action is believed to underlie drug reward and relapse (Wise, 1996a; Shalev *et al*, 2002; Wise and Gardner, 2002). The D_3 receptor has the highest binding affinity to endogenous DA of all known receptors (see for a review Sokoloff *et al*, 1992a; Levant, 1997), suggesting a vital role in the normal functioning of the mesolimbic DA system. D_3 receptor activation appears to enhance cocaine-induced reinforcement (Parsons *et al*, 1996). Congruently, it has been hypothesized that blockade of D_3 receptors may selectively block drug reward and relapse (Sokoloff *et al*, 1992a, b; Caine and Koob, 1993).

However, this hypothesis had been difficult to test due to the lack of D_3-receptor-selective ligands suitable for *in vivo* study. Recently, the D_3 partial agonist BP-897 (Pilla *et al*, 1999) and several D_3-selective antagonists such as SB-277011A, S33084, A-437203, and NGB 2904 (*N*-(4-[4-{2,3-dichlorophenyl}-1-piperazinyl]butyl)-3-fluorenylcarboxamide) have become available (Yuan *et al*, 1998; Stemp *et al*, 2000; Reavill *et al*, 2000; Millan *et al*, 2000), and some have

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been evaluated in animal models of addiction (for a review see Heidbreder *et al*, 2005). SB-277011A is the best characterized of these (see reviews by Le Foll *et al*, 2000; Schwartz *et al*, 2000; Remington and Kapur, 2001; Heidbreder *et al*, 2005). It significantly inhibits the rewarding effects of cocaine, heroin, and nicotine, as assessed by intravenous (i.v.) drug self-administration, intracranial brain stimulation reward (BSR), and conditioned place preference in rats (Vorel *et al*, 2002; Campos *et al*, 2003; Ashby *et al*, 2003; Gilbert *et al*, 2003). It also inhibits drug-seeking behavior as measured by second-order reinforcement and by cocaine-, nicotine-, or stress-triggered relapse to drug-seeking in the reinstatement model (Vorel *et al*, 2002; Andreoli *et al*, 2003; Di Ciano *et al*, 2003; Cervo *et al*, 2003; Xi *et al*, 2004). These data strongly support the potential of SB-277011A in the treatment of drug addiction. However, clinical development of SB-277011A has been terminated, due to its short half-life and poor bioavailability in primates (Austin *et al*, 2001; Heidbreder *et al*, 2005).

Another novel highly selective DA D_3 receptor antagonist, NGB 2904, has been synthesized (Yuan *et al*, 1998). This compound has structural similarity to BP-897 (Pilla *et al*, 1999; Wood *et al*, 2000; Wicke and Garcia-Ladona, 2001), and binds with high affinity to cloned primate D_3 receptors (K_i 1.4 nM) (Yuan *et al*, 1998; Robarge *et al*, 2001). NGB 2904 is reported to have 155-fold selectivity for primate D_3 over primate D_2 receptors, and >800-fold selectivity for rat D_3 *vs* D_2 receptors (Yuan *et al*, 1998; Newman *et al*, 2003). Also, it has >5000-fold selectivity over D_1, D_4, and D_5 receptors and 200- to 600-fold selectivity over α_1 and $5HT_2$ receptors (Yuan *et al*, 1998). Preliminary pharmacokinetic studies show that NGB 2904 is orally bioavailable and can penetrate the blood–brain barrier (unpublished data from GlaxoSmithKline by Heidbreder *et al*, personal communication). However, only limited behavioral evaluation of NGB 2904 in animal models of drug reward and relapse has been reported.

Therefore, in this study, we sought to behaviorally evaluate NGB 2904 in animal models of drug reward and relapse by determining: (1) whether blockade of D_3 receptors by NGB 2904 alters cocaine's rewarding effects, measured by i.v. cocaine self-administration under both fixed-ratio (FR) and progressive-ratio (PR) reinforcement schedules, and by BSR; (2) whether NGB 2904 itself has reinforcing effects, as assessed by NGB 2904 replacement for cocaine in self-administration, and as assessed by the effects of NGB 2904 itself on BSR; and (3) whether NGB 2904 alters cocaine-triggered reinstatement (relapse) of drug-seeking behavior, as compared to natural reinforcer-triggered reinstatement of goal-seeking behavior.

MATERIALS AND METHODS

Animals

Experimentally naïve male Long–Evans rats (Charles River Laboratories, Raleigh, NC, USA) weighing 250–300 g were used for all experiments. They were housed individually in a climate-controlled animal colony room on a reversed light–dark cycle (lights on at 19 00 hours, lights off at 0700 hours) with free access to food and water. The animals were maintained in a facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All experimental procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Academy of Sciences, 1996) and were approved by the Animal Care and Use Committee of the National Institute on Drug Abuse of the US National Institutes of Health.

Experimental Set 1: Cocaine Self-Administration

Surgery. All animals were prepared for experimentation by surgical catheterization of the right external jugular vein. The venous catheters were constructed of microrenathane (Braintree Scientific Inc., Braintree, MA, USA), and catheterization was performed under sodium pentobarbital anesthesia (65 mg/kg intraperitoneal (i.p.)) with aseptic surgical technique. After exiting the jugular, the catheter passed subcutaneously to the top of the skull, where it exited into a connector (a modified 24 G cannula; Plastics One, Roanoke, VA, USA) mounted to the skull with jeweler's screws and dental acrylic. During experimental sessions, the catheter was connected to the injection pump via tubing encased in a protective metal spring from the head-mounted connector to the top of the experimental chamber. To help prevent clogging, the catheters were flushed daily with a gentamicin–heparin–saline solution (30 IU/ml heparin; ICN Biochemicals, Cleveland, OH, USA).

Apparatus. The i.v. self-administration experiments were conducted in operant response test chambers ($32 \times 25 \times 33$ cm) from MED Associates Inc. (Georgia, VT, USA). Each test chamber had two levers located 6.5 cm above the floor, one active and one inactive. Depression of the active lever activated the infusion pump; depression of the inactive lever was counted but had no consequence. A cue-light and a speaker were located 12 cm above the active lever. The house light was turned on at the start of each 3 h test session. When the animal performed a lever-press that resulted in a drug infusion, it was exposed to two drug-paired environmental cues: a cue-light and a cue-sound (tone) that lasted for the duration of the infusion. Scheduling of experimental events and data collection were accomplished using MED Associates software.

General procedure. After recovery from surgery, each rat was placed into a test chamber and allowed to lever-press for i.v. cocaine (1 mg/kg/injection) delivered in 0.08 ml over 4.6 s, on an FR1 reinforcement schedule. During the 4.6 s injection time, additional responses on the active lever were recorded but did not lead to additional infusions. Each session lasted 3 h. The FR1 reinforcement schedule was used for 3–5 days until stable cocaine self-administration was established. The initial cocaine dose of 1 mg/kg/infusion was chosen on the basis of our previous experience that this dose produces the most rapid and facile acquisition of cocaine self-administration behavior. Subsequently, subjects were randomly assigned to one of the following four experiments: (1) cocaine self-administration under an FR2 reinforcement schedule, (2) cocaine self-administration under a PR reinforcement schedule, (3) NGB 2904 or saline replacement testing in experienced cocaine self-administer-

ing rats, or (4) cocaine-triggered reinstatement of drug-seeking behavior. In all experiments, NGB 2904 was given 30 min prior to testing because preliminary data showed that NGB 2904's effects occurred approximately 30 min after systemic administration.

Cocaine self-administration under FR2 reinforcement. After transition from FR1 reinforcement, subjects ($n = 10$) were allowed to continue cocaine (0.5 mg/kg/infusion) self-administration under FR2 reinforcement until the following criteria for stable cocaine-maintained responding were met: $<10\%$ variability in inter-response interval and $<10\%$ variability in the number of presses on the active lever for at least 3 consecutive days. The dose of cocaine was chosen on the basis of previous findings that rats self-administering cocaine at 0.5 mg/kg/infusion display highly stable self-administration behavior. In addition, previous studies have shown that 0.5 or 1 mg/kg/infusion cocaine lies within the range of the descending limb of the cocaine dose–response self-administration curve, where stable and reliable dose-dependent effects have been observed (Weissenborn *et al*, 1998; Parsons *et al*, 1998; Xi *et al*, 2005). Furthermore, we chose 0.5 mg/kg rather than 1 mg/kg of cocaine in order to increase the work demand (ie lever presses) of the animals for the same amount of drug intake. In our previous experience, this approach increases the sensitivity of measuring changes in drug-taking or drug-seeking behavior. To avoid cocaine overdose during the self-administration period, each animal was limited to a maximum of 50 cocaine injections per session. After stable rates of responding were established, each subject randomly received one of four doses of NGB 2904 (0.1, 1, 5, and 10 mg/kg i.p.) or vehicle (1 ml of 25% 2-hydroxypropyl-β-cyclodextrin solution) 30 min prior to the test session. Animals then received an additional 5–7 days of self-administration of cocaine alone until the baseline response rate was re-established prior to testing the next dose of NGB 2904. The order of testing for the various doses of NGB 2904 was counterbalanced according to a Latin square design.

Cocaine self-administration under PR reinforcement. Initial cocaine self-administration under FR1 and FR2 reinforcement was identical to that outlined above. After stable cocaine self-administration under FR2 reinforcement was established, the subjects were switched to cocaine self-administration (0.5 mg/kg/injection) under a PR schedule, during which the work requirement of lever presses needed to receive a single i.v. cocaine infusion was progressively raised within each test session (see details in Richardson and Roberts, 1996) according to the following PR series: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, and 603 until the break-point was reached. The break-point was defined as the maximal workload (ie number of lever presses) completed for the last cocaine infusion prior to a 1-h period during which no infusions were obtained by the animal. Animals were allowed to continue daily sessions of cocaine self-administration under PR reinforcement conditions until day-to-day variability in break-point fell within 1–2 ratio increments for 3 consecutive days. Once a stable break-point was established, subjects were assigned to four subgroups to determine the effects of three different doses of NGB 2904 (0.1, 1, and 5 mg/kg i.p.) or vehicle (1 ml 25% 2-hydroxypropyl-β-cyclodextrin solution) on PR break-point for cocaine self-administration. Since it is relatively difficult to reachieve basal break-point levels after each drug test, we chose to use a between-subjects design rather than a within-subjects design for this experiment.

NGB 2904 or saline self-administration in rats formerly self-administering cocaine. After a stable pattern of daily cocaine self-administration (0.5 mg/kg/infusion) under FR2 reinforcement was established for at least 3 consecutive days, the animals were divided into five groups ($n = 8$ each): (1) cocaine (0.5 mg/kg/infusion) was available for self-administration on the following days in the usual 3-h test sessions; (2) cocaine was replaced by NGB 2904 (0.1 mg/kg/infusion); (3) cocaine was replaced by NGB 2904 (0.5 mg/kg/infusion); (4) cocaine was replaced by heroin (0.05 mg/kg/infusion); and (5) cocaine was replaced by saline (0.08 ml/infusion). Since animals might take several days to support self-administration for a novel reinforcer, each replacement test was repeated for 3–5 days. The doses of NGB 2904 were chosen on two grounds. First, NGB 2904's maximum solubility in the 5% 2-hydroxypropyl-β-cyclodextrin solution used as vehicle in this experiment is approximately 3 mg/ml, making 0.5 mg/kg/infusion the maximum feasible unit dose. Second, the cumulative i.v. NGB 2904 dose within the initial 30 min (5–8 infusions $\times$ 0.1 or 0.5 mg/kg/infusion, see Figure 2) was approximately 0.5–4 mg/kg, which is higher than the i.p. doses (0.1–5 mg/kg) of NGB 2904 found to be effective in the self-administration, BSR, and reinstatement of drug-seeking experiments detailed below, making failure to self-administer an *a fortiori* finding. Heroin and saline were chosen as positive and neutral reinforcer controls.

Experimental Set 2: Electrical Brain Stimulation Reward (BSR)

Surgery. Under the same anesthesia as used in Experimental Set 1, rats were placed in a stereotaxic frame, and a unilateral monopolar stainless-steel stimulating electrode (Plastics One, Roanoke, VA, USA) was placed into the lateral hypothalamus using standard aseptic surgical and stereotaxic techniques. The implant coordinates for the tips of the electrodes were AP $= -2.56$, ML $= \pm 1.9$, and DV $= -8.6$, according to the rat brain stereotaxic Atlas of Paxinos and Watson (1998). The electrode was attached to the skull with jeweler's screws and dental acrylic. A wire leading from the electrode was wrapped around a skull screw to serve as a current return.

Apparatus. The experiments were conducted in standard MED Associates operant chambers ($32 \times 25 \times 33$ cm). Each operant chamber had a lever located 6.5 cm above the floor, connected to an electrical stimulator.

General procedure. The general procedures for electrical BSR were the same as we have reported previously (Vorel *et al*, 2002; Hayes *et al*, 2003). Briefly, after 7 days of recovery from surgery, rats were allowed to self-train

(autoshape) to lever-press for rewarding BSR. Each press on the lever resulted in a 500-ms train of 0.1-ms rectangular cathodal pulses through the electrode in the rat's lateral hypothalamus, followed by a 500 ms 'timeout' in which further presses did not produce brain stimulation. The initial stimulation parameters were 72 Hz and 200 μA. If the animal did not learn to lever-press, the stimulation intensity was increased daily by 50 μA until the animal learned to press (45–60 responses/30 s) or a maximum of 800 μA was reached. Animals that did not lever-press at 800 μA or in which the stimulation produced unwanted effects (eg gross head or body movements, spinning, vocalization, or jumping) were removed from the experiment.

Rate-frequency BSR procedure. Following establishment of lever-pressing for BSR, animals were presented with a series of 16 different pulse frequencies, ranging from 141 to 25 Hz in descending order. At each pulse frequency, animals responded for two 30-s time periods ('bins'), following which the pulse frequency was decreased by 0.05 log units. Following each 30-s bin, the lever retracted for 5 s. Throughout the experiments, animals were run for three sessions a day. Response rate for each frequency was defined as the mean number of lever responses during two 30-s bins. Since lever-pressing behavior was variable during the first session (the 'warm-up' session), but was stable during the second and third sessions, the data from the first session were discarded, and the data from the second and third sessions were designated as the baseline session data and test session data, respectively. The BSR threshold (θ_0) was defined as the minimum frequency at which the animal responded for rewarding stimulation.

Testing the effects of cocaine and/or NGB 2904 on BSR. Once a baseline θ_0 value was achieved ($<15\%$ variation in θ_0 over 5 continuous days), the effects of cocaine and/or NGB 2904 on BSR were assessed. On test days, animals randomly received one of three different doses of NGB 2904 (0.1, 1, and 5 mg/kg i.p.) or vehicle (1 ml 25% 2-hydroxypropyl-β-cyclodextrin) 30 min prior to a cocaine injection (1, 2, or 10 mg/kg i.p.). After each test, animals received an additional 5–7 days of BSR restabilization until a new baseline θ_0 was established. The order of testing for various doses of NGB 2904 was counterbalanced according to a Latin square design. The effect of NGB 2904 on cocaine-enhanced BSR was evaluated by comparing cocaine-induced alterations in θ_0 value in the presence or absence of each dose of NGB 2904 pretreatment.

Experimental Set 3: Reinstatement of Drug-Seeking Behavior

Surgery, apparatus, and general procedure. The surgery, apparatus, and general procedure to establish stable cocaine-taking behavior were the same as in Experimental Set 1, above.

Extinction and testing for reinstatement. After stable cocaine self-administration was established, animals were exposed to extinction conditions, during which cocaine was replaced by saline, and the cocaine-associated cue-light and tone were turned off. Active lever-pressing led only to saline infusion. Daily 3 h extinction sessions for each rat continued until that rat lever-pressed <10 times per 3 h session for at least 3 consecutive days. After successful achievement of extinction, animals were divided into four groups for reinstatement testing. On the reinstatement test day, each group of rats received either the vehicle (25% 2-hydroxypropyl-β-cyclodextrin) or one dose of NGB 2904 (0.1, 1, and 5 mg/kg i.p.). At 30 min after vehicle or NGB 2904 administration, all rats were given a priming injection of cocaine (10 mg/kg i.p.) immediately before the reinstatement testing began. During the reinstatement test, the conditions were identical to those in extinction sessions. Cocaine-induced active lever-pressing responses (reinstatement) were recorded, although these lever-pressing responses did not lead to either cocaine infusions or presentation of the conditioned cues. Reinstatement test sessions lasted 3 h.

Sucrose-plus-cue-triggered reinstatement of sucrose-seeking behavior. The procedures for oral sucrose self-administration, extinction, and reinstatement testing were identical to the procedures used in the cocaine-triggered reinstatement test above, except for the following minor differences: (1) no surgery was carried out on the rats in this experiment; (2) active lever presses led to delivery of 0.1 ml of 5% sucrose solution into a liquid food tray on the operant chamber wall; and (3) reinstatement was triggered initially by one to two 'free' sucrose deliveries, and subsequent lever presses led to the presentation of the conditioned cue-light and tone. Since sucrose-triggered reinstatement is significantly weaker than cocaine-triggered reinstatement, we chose to use cues plus sucrose priming to facilitate reinstatement of sucrose-seeking behavior.

Drugs

Cocaine HCl or heroin HCl (Sigma Chemical Co., Saint Louis, MO, USA) was dissolved in physiological saline. NGB 2904 was synthesized as reported (Yuan *et al*, 1998) in the Medicinal Chemistry Section, Medications Discovery Research Branch, Intramural Research Program, National Institute on Drug Abuse. 2-Hydroxypropyl-β-cyclodextrin (25%) (Sigma/RBI, St Louis, MO) was used as vehicle for i.p. injections. To decrease injection resistance and solution osmotic pressure, 5% 2-hydroxypropyl-β-cyclodextrin was used as vehicle for i.v. NGB 2904 self-administration. We have previously observed no differences in behavioral effects between 5 and 25% 2-hydroxypropyl-β-cyclodextrin.

Data Analyses

All behavioral data are presented as means ($\pm$SEM), and were subjected to standard univariate or multivariate statistical analyses (Winer, 1962; Kirk, 1982). After subjecting data to preliminary testing to assure homogeneity of variance by Bartlett's (1937) test and to assure that other mathematical requirements for parametric analyses were met, one-way analysis of variance (ANOVA) was used to analyze the data reflecting the effects of NGB 2904 on PR cocaine self-administration, and the data on cocaine- or sucrose-triggered reinstatement of goal-seeking behavior.

One-way ANOVA for repeated measures was used to analyze the effects of NGB 2904 on FR2 cocaine self-administration and on cocaine-enhanced BSR. Two-way ANOVA with repeated measures on one factor was used to analyze the data reflecting the ability of NGB 2904, heroin, or saline to sustain self-administration. Post-ANOVA individual group comparisons were carried out using the Tukey (a) statistical procedure (also known as the Tukey honestly significant difference procedure).

RESULTS

Experimental Set 1: Cocaine Self-Administration

Effects of NGB 2904 on cocaine self-administration under FR2 reinforcement. NGB 2904 (0, 0.1, 1, 5, or 10 mg/kg i.p.) administered 30 min prior to the beginning of daily cocaine self-administration sessions had no significant effect on cocaine self-administration behavior at a unit cocaine reinforcement dose of 0.5 mg/kg/infusion compared to vehicle-treated animals (data not shown). One-way ANOVA for repeated measures over the NGB 2904 dose range revealed no statistically significant effect of NGB 2904 on cocaine self-administration (ie number of cocaine infusions) under FR2 reinforcement conditions ($F_{4,29} = 0.54$; $p = 0.71$).

Effects of NGB 2904 on cocaine self-administration under PR reinforcement. Figure 1a shows representative individual response records for cocaine self-administration under PR reinforcement conditions after vehicle or NGB 2904 administration in the same animal, illustrating a PR break-point (ie completed lever presses for the last cocaine infusion) of 95 for cocaine self-administration after vehicle administration (Figure 1a, upper trace) and a significantly lower PR break-point of 32 for cocaine self-administration after 5 mg/kg NGB 2904 administration (Figure 1a, lower trace). Figure 1b and c illustrate the group data for the observed NGB 2904-induced decrease in PR break-point itself ($F_{3,28} = 6.07$, $P = 0.003$) and the percent change in break-point ($F_{3,28} = 16.42$, $P < 0.001$). NGB 2904 significantly lowered the break-point for cocaine self-administration behavior reinforced under PR conditions. Individual group comparisons using the Tukey (a) statistical test revealed a statistically significant difference between PR break-point levels for cocaine self-administration after vehicle *vs* after 1 mg/kg NGB 2904 ($q = 4.96$, $p < 0.01$ for Figure 1b; $q = 7.76$, $p < 0.001$ for Figure 1c), and after

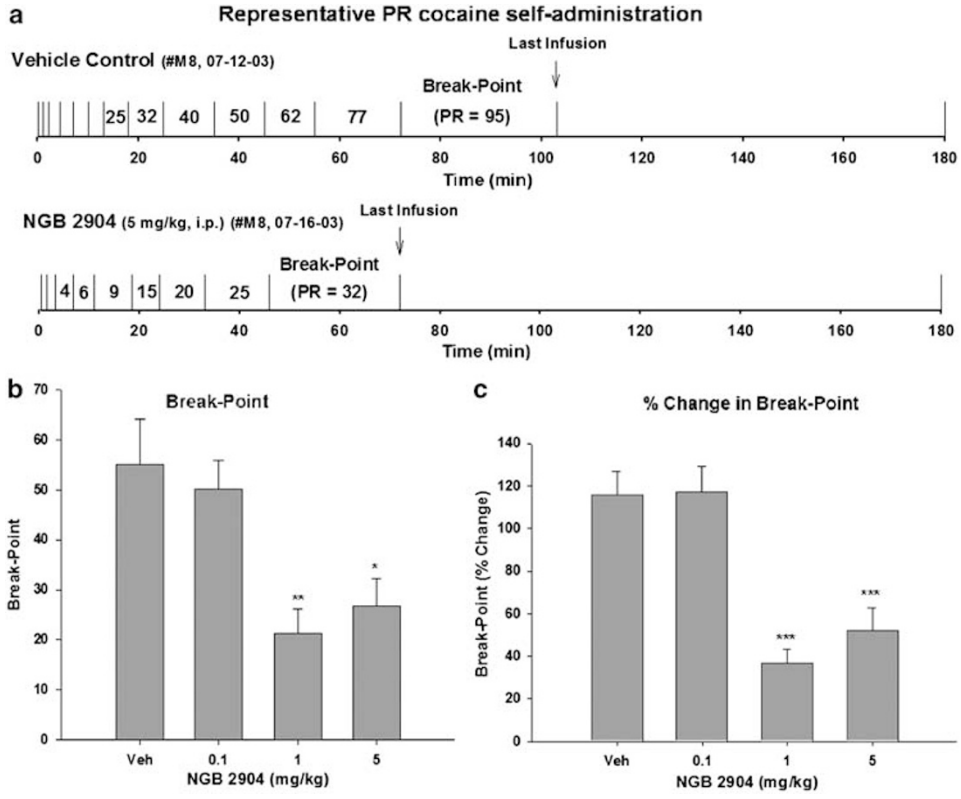

Figure 1 Effect of NGB 2904 on cocaine self-administration under PR reinforcement conditions. (a) Representative records of an individual animal illustrating a reduction in the PR break-point for cocaine self-administration from 95 after vehicle (1 ml 25% 2-hydroxypropyl-β-cyclodextrin i.p.; upper trace) to 32 after NGB 2904 (5 mg/kg i.p., 30 min prior to test; lower trace) pretreatment. Each vertical line indicates a cocaine infusion (0.5 mg/kg/infusion). The number between the vertical lines indicates the work demand (progressively increased PR ratio, that is, number of lever presses) for a subsequent cocaine infusion. The PR break-point was defined as the completed work requirement (lever presses) to receive the last cocaine infusion. (b) Depicts the changes in break-point itself, and (c) depicts the percent changes in PR break-point for cocaine self-administration after pretreatment with NGB 2904 (0.1, 1, or 5 mg/kg) or vehicle (25% 2-hydroxypropyl-β-cyclodextrin) on test day. One-way ANOVA revealed that NGB 2904 produced a statistically significant reduction in PR break-point for cocaine self-administration. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, individual group comparisons using the Tukey (a) statistic, when compared to the vehicle (Veh) pretreatment group.

vehicle *vs* after 5 mg/kg NGB 2904 ($q = 4.17$, $p < 0.05$ for Figure 1b; $q = 6.26$, $p < 0.001$ for Figure 1c).

Ability of NGB 2904 itself to sustain self-administration. Figure 2 shows the results of the replacement tests with NGB 2904 (0.1 or 0.5 mg/kg/infusion), heroin, cocaine, or saline in five separate groups of rats ($n = 8$ each) already experienced and displaying behaviorally stable cocaine self-administration under FR2 reinforcement conditions. Each substitution drug was tested repeatedly for 3–5 days. Figure 2a–c show representative single records of NGB 2904 (0.1 and 0.5 mg/kg/infusion) or heroin (0.05 mg/kg/infusion) replacement for cocaine. NGB 2904 at either dose failed to sustain a stable pattern of self-administration. In fact, the self-administration behavior underwent gradual extinction over the 3-h test period (Figure 2d). This pattern of extinction was essentially identical to that seen when saline (0.08 ml/infusion) was substituted for cocaine (Figure 2d). In contrast, both cocaine and heroin maintained self-administration behavior (Figure 2c and d), showing the typical loading phase of increased drug self-administration during the first 20 min of self-administration opportunity, followed by stable cocaine or heroin self-administration thereafter (Figure 2d). Two-way ANOVA for repeated measures over time revealed a significant main effect of substituting NGB 2904, cocaine, heroin, or saline for cocaine ($F_{4,27} = 64.86$, $p < 0.001$), a significant main effect over time ($F_{8,32} = 35.31$, $p < 0.001$), and a significant time × drug interaction effect ($F_{32,216} = 1.73$, $p = 0.012$). Individual group comparisons revealed statistically significant differences between drug-taking behavior for cocaine and drug-taking behavior for 0.1 mg/kg NGB 2904 ($q = 17.39$, $p < 0.001$), for 0.5 mg/kg NGB 2904 ($q = 17.93$, $p < 0.001$), for heroin ($q = 11.76$, $p < 0.001$), or for saline ($q = 18.68$, $p < 0.001$). Individual group comparisons revealed statistically significant differences between drug-taking behavior for heroin and drug-taking behavior for 0.1 mg/kg NGB 2904 ($q = 5.08$, $p < 0.05$), for 0.5 mg/kg NGB 2904 ($q = 4.39$, $p < 0.05$), or for saline ($q = 5.06$, $p < 0.05$). Further group comparisons revealed that there were *no*

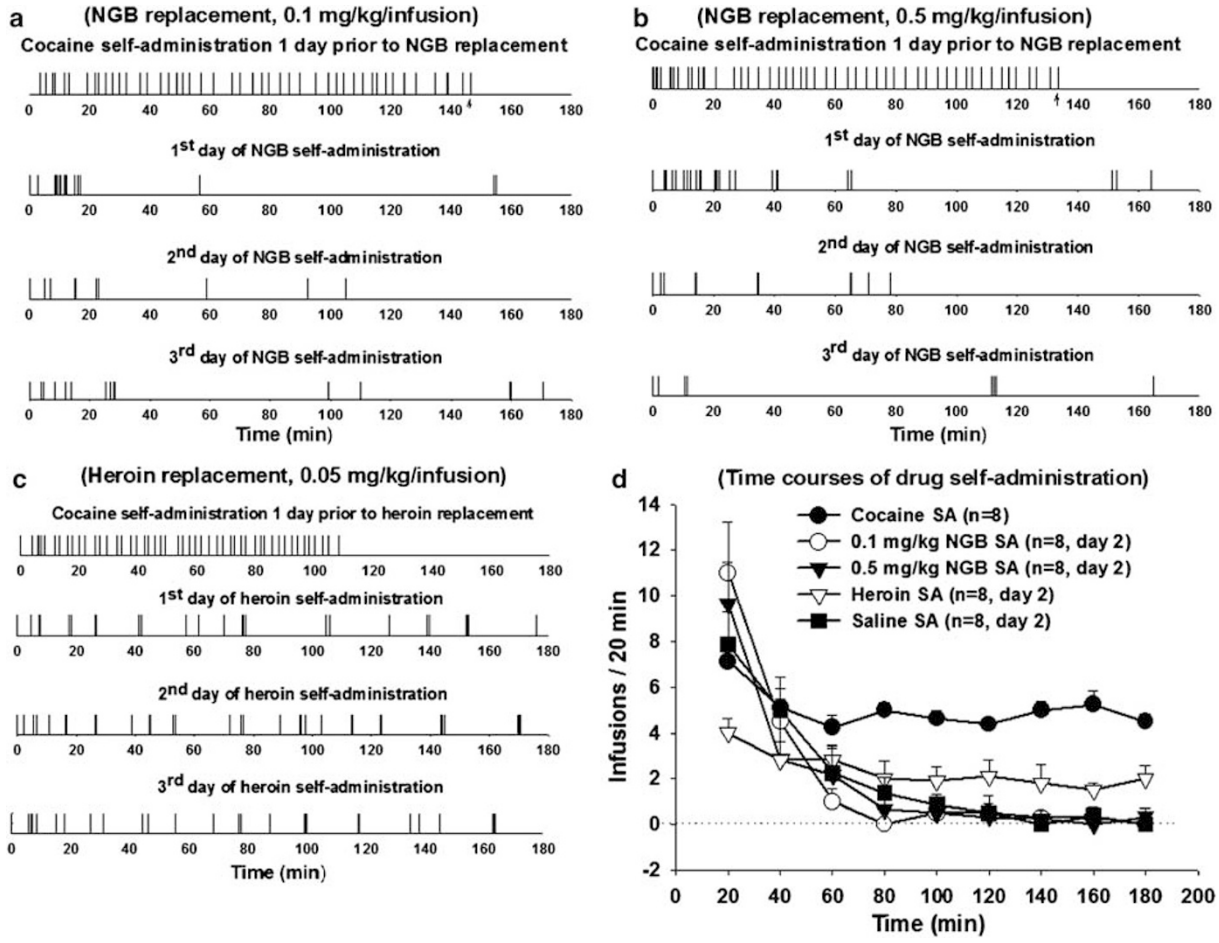

Figure 2 Effect of substituting NGB 2904, heroin, or saline for cocaine in animals proficient at cocaine self-administration behavior. (a–c) Representative event records of drug infusions (each vertical line represents one drug infusion), illustrating that NGB 2904 (0.1 or 0.5 mg/kg/infusion; a and b) failed to sustain, but heroin (0.05 mg/kg/infusion; c) did sustain drug self-administration behavior during three repeated days of substitution testing for cocaine. (d) Time courses of mean drug-taking behavior by 20 min intervals for 0.5 mg/kg cocaine (●—●), 0.1 mg/kg NGB 2904 (○—○), 0.5 mg/kg NGB 2904 (▼—▼), 0.05 mg/kg heroin (▽—▽), or saline (■—■) on the second substitution test day. Two-way ANOVA for repeated measures over time revealed statistically significant differences in drug-taking behavior between cocaine and NGB 2904 (0.1 or 0.5 mg/kg/infusion), between cocaine and heroin, between cocaine and saline, between heroin and NGB 2904, and between heroin and saline substitution groups. However, there were no significant differences in drug-taking behavior between any dose of NGB 2904 and the saline substitution group. Further, the extinction-like pattern of responding (a, b, and d) after substitution of NGB 2904 for cocaine suggests that NGB 2904 itself has no reinforcing effect. SA, self-administration; NGB, NGB 2904.

statistically significant differences between drug-taking behavior for NGB 2904 (0.1 or 0.5 mg/kg) and drug-taking behavior for saline at any 20-min test point over the entire 3-h test period (Figure 2d).

Experimental Set 2: Electrical BSR

Effects of NGB 2904 on cocaine-enhanced BSR. As shown in Figure 3 and as reported previously (Bauco and Wise, 1997; Gilliss *et al*, 2002), systemic administration of cocaine (1, 2, and 10 mg/kg i.p.) produced significant (and dose-dependent) enhancement of BSR ($F_{2,10} = 11.01$, $p = 0.003$) (Figure 3a, right panel), manifested as a decrease in BSR threshold (θ_0 value). NGB 2904 alone (1 and 5 mg/kg i.p.) had no effect on BSR ($F_{2,14} = 1.345$, $p = 0.292$) (Figure 3a, left panel). Pretreatment with NGB 2904 (0.1, 1 mg/kg, but not 5 mg/kg) significantly inhibited the enhanced BSR produced by 2 mg/kg, but not by 10 mg/kg, of cocaine (Figure 3b and c). One-way ANOVA for repeated measures revealed a statistically significant main effect of NGB 2904 for the 2 mg/kg cocaine treatment group ($F_{3,21} = 4.57$, $p = 0.013$) (Figure 3b), but not for the 10 mg/kg cocaine treatment group ($F_{3,15} = 0.18$, $p = 0.91$) (Figure 3c). Individual group comparisons revealed statistically significant differences in the effect on cocaine-enhanced BSR between vehicle and 0.1 mg/kg NGB 2904 ($q = 4.68$, $p = 0.016$) and between vehicle and 1 mg/kg NGB 2904 ($q = 4.37$, $p = 0.026$), but not between vehicle and 5 mg/kg NGB 2904 treatment groups ($q = 3.06$, $p = 0.066$) (Figure 3b).

Experimental Set 3: Reinstatement of Drug-Seeking Behavior

Effects of NGB 2904 on cocaine- or sucrose-plus-sucrose-cue-triggered reinstatement of cocaine-seeking or sucrose-seeking behavior. Figure 4 shows the total numbers of active lever presses observed during the last session of cocaine self-administration, during the last session of extinction, and during the reinstatement test session in the four different NGB 2904 dose groups. A single, noncontingent cocaine injection (10 mg/kg i.p.) produced robust reinstatement of extinguished operant behavior previously reinforced by i.v. cocaine infusions. Pretreatment with NGB 2904 produced a significant attenuation of this cocaine-triggered reinstatement of drug-seeking ($F_{3,24} = 4.00$, $p = 0.019$). Individual group comparisons revealed a statistically significant difference in cocaine-induced reinstatement of cocaine-seeking behavior between vehicle and 1 mg/kg NGB 2904 ($q = 4.16$, $p = 0.034$) and between vehicle and 5 mg/kg NGB 2904 ($q = 4.24$, $p = 0.03$), but not between vehicle and 0.1 mg/kg NGB 2904 ($q = 3.34$, $p = 0.113$). There was no difference in inactive lever responses between vehicle and any dose of NGB 2904 (data not shown). Finally, as shown in Figure 4b, NGB 2904 (1 or 5 mg/kg i.p.) had no effect on sucrose-plus-sucrose-cue-triggered reinstatement of sucrose-seeking behavior ($F_{2,15} = 0.038$, $p = 0.96$).

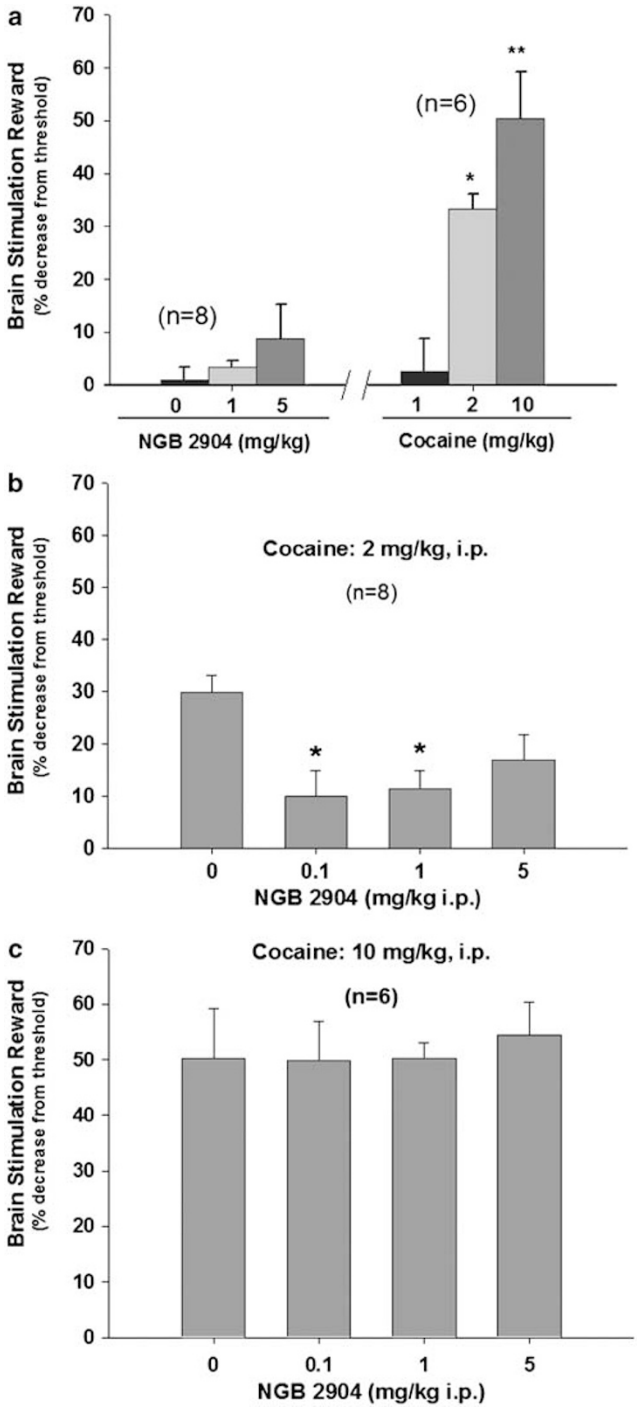

Figure 3 Effects of cocaine and NGB 2904 on electrical brain stimulation reward (BSR). (a) NGB 2904 alone (1, 5 mg/kg i.p.) had no significant effect on BSR, while cocaine (1, 2, and 10 mg/kg i.p.) produced a dose-dependent enhancement in BSR, as assessed by a statistically significant decrease in stimulation threshold for brain reward. (b) The mean percentage change in BSR threshold produced by 2 mg/kg cocaine in the absence or presence of NGB 2904 (0.1, 1, and 5 mg/kg i.p., 30 min prior to test). (c) The ineffectiveness of the same doses of NGB 2904 on 10 mg/kg cocaine-enhanced BSR. $^*p < 0.05$, $^{**}P < 0.01$, individual group comparisons using the Tukey (a) statistic, when compared with vehicle or baseline.

DISCUSSION

The present study, for the first time, demonstrates that systemic administration of the highly selective DA D_3 receptor antagonist NGB 2904 attenuates cocaine's rewarding effects, as assessed by both the PR self-administration and electrical BSR paradigms. In the reinstatement animal

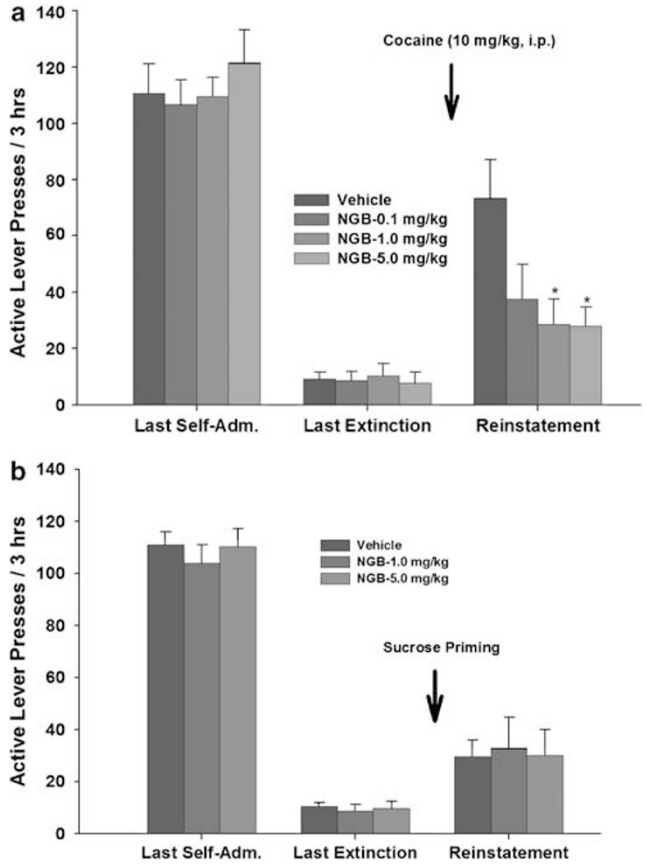

Figure 4 Effects of NGB 2904 on cocaine- or sucrose-plus-sucrose-cue-triggered reinstatement of reinforcer-seeking behavior. (a) Illustrates that pretreatment with NGB 2904 (0.1, 1, and 5 mg/kg i.p., 30 min prior to test) significantly inhibited cocaine-triggered reinstatement of drug-seeking behavior in rats extinguished from daily cocaine self-administration. $^*p < 0.05$, when compared with the vehicle pretreatment group. (b) Illustrates the ineffectiveness of NGB 2904 (1 and 5 mg/kg i.p.) on sucrose-plus-sucrose-cue-triggered reinstatement of sucrose-seeking behavior. Last Self-Adm, last session of cocaine self-administration; Last Extinction, last session of extinction 24 h before reinstatement testing.

model of relapse, NGB 2904 also significantly inhibited cocaine-triggered drug-seeking behavior, but not sucrose-plus-sucrose-cue-triggered sucrose-seeking behavior. NGB 2904 itself had no reinforcing effect in rats previously experienced at cocaine self-administration, and did not by itself produce any significant effect on BSR. Together, these data support the hypothesis that DA D_3 receptors play a critical role in acute cocaine-induced reinforcement and reinstatement (relapse) of drug-seeking behavior. The data also provide additional support for the potential utility of NGB 2904 or other highly selective DA D_3 antagonists in the treatment of cocaine dependence.

NGB 2904's Inhibition of Cocaine Self-Administration under PR Reinforcement Conditions

Drug self-administration paradigms in animals offer the most face valid models of drug-taking behavior in humans (Wise and Gardner, 2004). There are many self-administration paradigms at the animal level, of which the FR reinforcement variant is the most widely used. In this model, the addictive drug is readily available to animals under low-effort (low work demand) and high-payoff (high unit dose of drug) conditions. In the PR paradigm, the highest completed work demand for a reward (the PR break-point) is considered a measure of rewarding efficacy (Roberts, 1989; Roberts *et al*, 1989; Richardson and Roberts, 1996). In the present study, we found that NGB 2904 had no significant effect on cocaine self-administration under FR2 reinforcement conditions, but significantly lowered the break-point for cocaine self-administration under PR reinforcement conditions. This is congruent with previous findings that the selective D_3 receptor antagonist SB-277011A inhibits cocaine self-administration under a PR reinforcement schedule, but not under a low-cost high-payoff FR reinforcement schedule (Di Ciano *et al*, 2003; Gilbert *et al*, 2003; Gál and Gyertyán, 2003; Xi *et al*, 2005). There are three possible explanations for the ineffectiveness of NGB 2904 on FR cocaine self-administration. First, the FR2 reinforcement schedule demands less work and provides a much higher cumulative cocaine-dose payoff than does the PR reinforcement schedule. Thus, the stronger rewarding effects produced by the higher cumulative dose of cocaine may overcome NGB 2904's antagonism of cocaine's effect. Second, animals may compensate for NGB 2904's action by increasing their drug intake or their self-administration rate under FR2 conditions. We consider this second possibility to be unlikely, as we did not observe such a compensatory increase in cocaine intake in the present study. Although we limited the number of maximal drug infusions, which may have masked a compensatory response, we saw neither a significant difference in the total time spent to gain the maximal 50 infusions nor a change in cocaine self-administration rate in the presence or absence of NGB 2904 pretreatment (data not shown). Third, it is possible that FR cocaine self-administration is relatively insensitive to changes in reinforcement efficacy, as suggested by several authorities in the field (eg Roberts, 1989; Katz, 1990; Arnold and Roberts, 1997), who note that FR reinforcement measures the *fact* of drug reinforcement, but not the *degree* of reinforcing efficacy (see also Gardner, 2000; Wise and Gardner, 2004; Xi *et al*, 2005).

In contrast to FR reinforcement, the PR break-point shift paradigm is extremely sensitive to dose–response functions that reflect a given drug's reinforcing efficacy (Roberts *et al*, 1989; Roberts and Bennett, 1993; French *et al*, 1995; Arnold and Roberts, 1997; Stafford *et al*, 1998). Based on the assumption that high reward stimulates high motivation and high-effort behavior, the PR self-administration paradigm is believed to also measure motivation to self-administer addictive drugs (Richardson and Roberts, 1996; Arnold and Roberts, 1997; Stafford *et al*, 1998; Rowlett, 2000). Thus, the present finding that blockade of D_3 receptors by NGB 2904 significantly inhibits cocaine self-administration under PR reinforcement conditions suggests that NGB 2904 antagonizes cocaine's rewarding efficacy and therefore its incentive motivational properties. This finding is consistent with previous studies demonstrating that the selective D_3 receptor antagonist SB-277011A also inhibits cocaine-seeking behavior under PR and second-order reinforcement conditions, but not under FR reinforcement conditions (Di Ciano *et al*, 2003; Gilbert *et al*, 2003; Xi *et al*, 2005).

NGB 2904's Attenuation of Cocaine-Enhanced BSR

The electrical BSR paradigm is believed to measure a neural substrate of reward that summates with, and is highly sensitive to, drug-induced reward (Stein and Ray, 1960; Wise, 1996b; Wise and Gardner, 2004; Gardner, 2005). In the present study, cocaine dose-dependently decreased stimulation thresholds for brain reward, indicating summation or synergism between the reward provided by the electrical brain stimulation and the cocaine-induced reward (for further discussion of such summation effects, see Bauco and Wise, 1997; Gilliss *et al*, 2002). Pretreatment with NGB 2904 (0.1 and 1 mg/kg) significantly inhibited the enhanced BSR produced by 2 mg/kg cocaine. As shown in Figure 3, the apparent inhibitory effect produced by 5 mg/kg NGB 2904 on cocaine-enhanced BSR is not statistically significant, and the reasons for this are unclear. Since only those subjects who received all four different test doses were included in our data analysis, the relatively variable data generated from some of the animals may have contributed to this nonsignificant statistical result. However, we cannot exclude the possibility that high doses of NGB 2904 may produce other non-D_3 receptor-mediated effects that, in turn, may mask D_3 receptor-mediated inhibition of cocaine's enhancement of BSR. However, this seems unlikely because the same dose of NGB 2904 (5 mg/kg) inhibited cocaine self-administration under PR reinforcement conditions and also inhibited cocaine-triggered reinstatement of drug-seeking behavior.

The same dose range of NGB 2904 had no effects on the enhanced BSR produced by 10 mg/kg cocaine, suggesting that the antagonism by NGB 2904 of cocaine-enhanced brain reward is surmountable by increased cocaine dose. Two possible mechanisms may underlie such dependency on cocaine dose. Since D_3 receptors have the highest binding affinity to endogenous DA (Levant, 1997), we suggest that D_3 receptors may play a critical role in the regulation of reward tone under physiological or mildly elevated DA transmission conditions, such as those created by low doses of cocaine (see also Parsons *et al*, 1996). However, when extracellular DA levels are very high, for example, under high cocaine dose conditions, other DA receptor subtypes may be affected. Self *et al* (1996) have reported that activation of DA D_2 receptors enhances cocaine-induced drug-seeking behavior, an effect that would be congruent with an enhancement of cocaine-induced reward, as some evidence suggests that drug-seeking behavior is correlated with increased DA function in the nucleus accumbens (Stewart and Vezina, 1988; Ranaldi *et al*, 1999; Weiss *et al*, 2000; Di Ciano *et al*, 2001). Therefore, it is possible that at high cocaine doses, activation of other DA receptors may diminish the attenuation of cocaine-induced reward produced by D_3 receptor antagonism. Alternatively, high levels of extracellular DA produced by high doses of cocaine may bind to D_3 receptors in a competitive manner, attenuating the binding of NGB 2904 to the same D_3 receptors. This suggestion is consistent with our findings that NGB 2904 or SB-277011A had no effect on cocaine self-administration under continuous FR reinforcement conditions in which a high cumulative dose is easily built up, but significantly inhibited cocaine self-administration under PR reinforcement conditions in which a high cumulative dose of drug is difficult to achieve (Di Ciano *et al*, 2003; Gilbert *et al*, 2003). Similarly, BP-897 (a D_3 partial agonist whose antagonist properties may predominate; see Wood *et al*, 2000; Wicke and Garcia-Ladona, 2001) also fails to inhibit cocaine self-administration under FR reinforcement conditions, but significantly inhibits cocaine-seeking behavior under second-order reinforcement conditions (Pilla *et al*, 1999), under which the brain's DA response to the cocaine-associated cues that drive second-order reinforced drug-seeking behavior is substantially lower than to cocaine itself (ie approximately a 25% increase in accumbens DA produced by cocaine-associated cues (Gerasimov *et al*, 2001) *vs* approximately a 200–800% increase in accumbens DA produced by either exogenously or self-administered cocaine (Wise *et al*, 1995; Dewey *et al*, 1997)).

Noteworthily, the present data show that NGB 2904 itself produces neither a significant reward-like leftward shift nor a rightward shift in BSR rate-frequency functions. This is similar to our findings with SB-277011A (Campos *et al*, 2003, 2004). This intermodel, inter-D_3 antagonist consistency further supports the conclusion that highly selective DA D_3 receptor antagonists diminish cocaine's reinforcing efficacy or reward value.

NGB 2904's Inhibition of Cocaine-Triggered Reinstatement of Drug-Seeking Behavior

Drug reward and craving-driven relapse are believed to constitute critically important mechanistic substrates underlying addiction, with relapse believed to be well modeled at the laboratory animal level by cocaine-triggered reinstatement of cocaine-seeking behavior (Shalev *et al*, 2002). Using this animal relapse model, we found that pretreatment with NGB 2904 (0.1, 1, and 5 mg/kg) significantly attenuated cocaine-triggered reinstatement of cocaine-seeking behavior at doses that failed to reduce sucrose-plus-sucrose-cue-induced reinstatement of sucrose-seeking behavior, suggesting that NGB 2904-induced reduction in cocaine-seeking behavior is not caused by a generalized suppression of operant behavior. This finding is consistent with the previous finding that SB-277011A inhibits cocaine-triggered reinstatement of cocaine-seeking, but not food-triggered reinstatement of food-seeking (Vorel *et al*, 2002).

As noted above, NGB 2904 (1 and 5 mg/kg) significantly inhibited reinstatement (maximally by around 50%), but not the enhanced BSR produced by 10 mg/kg i.p. of cocaine. It has been reported that in rats undergoing reinstatement testing or extinction from cocaine self-administration, there is significant attenuation of cocaine-induced increases in extracellular DA in the nucleus accumbens (Neisewander *et al*, 1996; Mateo *et al*, 2005; but see Hooks *et al*, 1994). In contrast, electrical BSR *per se* appears to *enhance* nucleus accumbens DA (You *et al*, 2001). Thus, it is possible that cocaine plus electrical BSR may produce an additive or synergistic effect on DA efflux, and that, therefore, the same dose of cocaine may produce a stronger DA response in rats undergoing BSR than in rats undergoing reinstatement testing. Further, as we noted above, NGB 2904's antagonism of cocaine's actions may depend on endogenous DA levels, which in turn may explain why the same doses of NGB 2904 that produce a 50% inhibition of 10 mg/kg cocaine-triggered

reinstatement of drug-seeking behavior had no effect on 10 mg/kg cocaine-enhanced BSR.

Importantly, the presently observed reduction in cocaine self-administration or cocaine-triggered drug-seeking behavior caused by NGB 2904 seems unlikely to have resulted from impaired motor function, because NGB 2904, at the dose range tested, alters neither cocaine self-administration behavior under FR reinforcement (data not shown) nor sucrose-plus-sucrose-cue-triggered sucrose-seeking behavior (see Figure 4). It also has no effect on the enhancement of BSR produced by 10 mg/kg of cocaine (see Figure 3) or responding on the inactive lever in all of these operant behavioral experiments (data not shown).

NGB 2904's Selective Blockade of DA D_3 Receptors

A number of studies have shown that D_1- or D_2-preferring antagonists inhibit cocaine self-administration, cocaine-enhanced BSR, and cocaine-triggered reinstatement of drug-seeking behavior (Wilson and Schuster, 1972; de Wit and Wise, 1977; Koob *et al*, 1987; Bergman *et al*, 1990; Corrigall and Coen, 1991; McGregor and Roberts, 1993; Caine and Koob, 1994; Caine *et al*, 1995, 2002; Self *et al*, 1996; Awasaki *et al*, 1997; Ikemoto *et al*, 1997; Kita *et al*, 1999; Spealman *et al*, 1999; Khroyan *et al*, 2000, 2003; Hummel and Unterwald, 2002; Norman *et al*, 2002; Anderson *et al*, 2003; Sanchez *et al*, 2003; for reviews see Platt *et al*, 2002; Kapur and Mamo, 2003; Gorelick *et al*, 2004). Consequently, this raises the issue of whether the presently observed attenuating effects of NGB 2904 in these three behavioral paradigms might be attributable to D_1 or D_2 receptor-selective antagonism rather than to D_3 receptor-selective antagonism. We believe that is unlikely, because (1) multiple lines of *in vitro* evidence indicate that NGB 2904 is a highly selective D_3 receptor antagonist (Yuan *et al*, 1998; Robarge *et al*, 2001; Newman *et al*, 2003, 2005; see Introduction); (2) in the BSR paradigm, D_1- and D_2-preferring DA receptor antagonists *inhibit* brain reward functions, in a manner opposite to the brain reward *enhancement* produced by addictive drugs (Stein and Ray, 1960; Stein, 1962; Wise, 1982; Panagis and Spyraki, 1996; Gardner, 2005), while selective blockade of DA D_3 receptors by either NGB 2904 or SB-277011A does *not* alter electrical brain reward thresholds (Vorel *et al*, 2002; Campos *et al*, 2003, 2004); and finally (3) NGB 2904 does not significantly alter locomotor activity at the dose range tested in the present study (Newman *et al*, 2005), again unlike D_1 or D_2 receptor antagonists.

In conclusion, NGB 2904 attenuates cocaine's rewarding effects and selectively inhibits relapse to drug-seeking behavior, and has no apparent rewarding, aversive, or locomotor effects. Therefore, NGB 2904 or other highly selective D_3 antagonists may be promising as pharmacotherapeutic agents to treat cocaine abuse and may provide *in vivo* tools with which to further characterize the role of DA D_3 receptors in drug addiction.

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