Cocaine vaccines: Antibody protection against relapse in a rat model

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The efficacy of active immunization with the cocaine immunogen GNC-keyhole limpet hemocyanin (KLH) in preventing cocaine self-administration reinstatement was assessed in rats. An animal model of relapse was used where rats were trained to self-administer cocaine, subjected to a period of extinction by substituting the drug for saline, vaccinated, and re-exposed to cocaine. Compared with controls, animals immunized with GNC-KLH did not reinstate cocaine self-administration behavior when given a noncontingent cocaine infusion on two consecutive days. Upon double and triple infusions, 38–62% of vaccinated animals failed to reinstate as compared with full reinstatement in all control animals. Exposure to ad libitum cocaine reinstated baseline values in control animals and resulted in double to triple the baseline values of self-infusions in vaccinated animals, suggesting a partial antibody-mediated blockade of cocaine access to the central nervous system. This compensating effect was blocked by passive immunization pretreatment with the monoclonal IgG GNC92H2 in both vaccinated and control groups. To further assess the surmountability potential of GNC-KLH-induced antibody titer by cocaine self-administration, and the capacity of these titer to block the reinforcing effects of the drug, rats were tested at various doses of cocaine (0.015–0.5 mg/injection). Active immunization with GNC-KLH produced approximately an 8-fold rightward shift of the dose-effect function for cocaine. The results reported suggest that immunopharmacotherapy may offer a promising means to treat cocaine abuse by aiding in the prevention of relapse.

Cocaine abuse prevails as a major public health concern (1), causing direct adverse medical consequences (2) and contributing to grave societal problems (3). Despite substantial multidisciplinary research, an effective treatment against this illness remains elusive (4). Recently, immunopharmacotherapy was proposed as a promising means to treat cocaine abuse. By using the natural immune response of an organism, an anticocaine vaccine promotes the production of cocaine-specific antibodies that inhibit the passage of cocaine into the brain. This approach offers a drug-specific, peripheral means of cocaine blockade, thus circumventing the adverse secondary effects of pharmacotherapy.

A study from this laboratory showed that active immunization with GNC-keyhole limpet hemocyanin (KLH), a novel cocaine-KLH conjugate, suppressed the psychostimulant effects of cocaine in rats (5). Significantly, brain cocaine levels were found to be 80% lower in immunized rats compared with controls. Other groups reported immunopharmacological approaches to blunt the analgesic and reinforcing effects of the drug (6–8). However, these reports have raised concerns regarding the efficacy of the conjugate (9), antibody surmountability (10, 11), and the choice of schedules of reinforcement (11). These concerns must be addressed to place the potential therapeutic value of an anticocaine conjugate into relevant clinical context. In this regard, it is imperative to use an animal model of cocaine self-administration that allows the assessment of both the efficacy and the surmountability threshold of the immune-mediated response in preventing relapse.

Cocaine is known to have reinforcing qualities that contribute to rapid addiction and high rates of relapse after long periods of abstinence (12, 13). Among the many factors that lead to cocaine recidivism, re-exposure to the drug itself has been proven to be one of the most powerful in humans and animals (12, 14–16). Stewart and de Wit (16–18) have extensively investigated this phenomenon with cocaine and other drugs of abuse in rats by using a reinstatement procedure. In this assay, animals initially trained to self-administer drugs i.v. After the development of stable drug-taking behavior, lever pressing for drug infusions is extinguished by substituting saline for the drug. After extinction of lever pressing, the ability of a single, noncontingent administration of the training drug to elicit renewed responding is examined. Using this procedure, it has been demonstrated that a single “priming” infusion of the drug given by the experimenter elicits a period of renewed responding (19), and that this effect survives a long-term extinction of drug-taking behavior (20). Thus, the presence of drug in the body appears to enhance drug-related behavior in animals that returned to the environment where drug has in the past been available.

The present study examines the efficacy of active immunization with GNC-KLH (5) in preventing cocaine reinstatement, using a modified version of the aforementioned reinstatement procedure. Also, the resilience of the anticocaine immune response alone and in combination with passive immunization with the GNC-KLH-elicted monoclonal IgG GNC92H2 has been assessed by allowing ad libitum access to i.v. cocaine self-administration. In a separate study, the potency of GNC92H2 for cocaine antagonism was examined by testing a range of doses of the antibody in cocaine self-administering rats. Finally, the efficacy of active immunization with GNC-KLH in suppressing the reinforcing properties of cocaine was further evaluated by exposing vaccinated animals to different doses of cocaine to generate the typical inverted U-shaped dose–response function. In this behavioral assay, a displacement to the right of the dose–response function reflects surmountable antagonism by a treatment agent (21–23).

Materials and Methods

Animals. Male Wistar rats (n = 50; Charles River Breeding Laboratories), weighing 225–250 g on arrival, were housed in groups of 2–3 in a humidity- and temperature-controlled (22°C) vivarium on a 12-h light/dark cycle (lights on at 10 p.m.). All behavioral procedures were performed during the dark phase. All experiments were conducted by using a between-subject design except for the passive immunization dose–response study, where a within-subjects design was used. Data are presented from all animals that completed the experimental designs (41 rats). After the initial week of operant training (see the next
section) animals were provided with food and water ad libitum throughout the study. All procedures were conducted in strict adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Self-Administration Training. Rats were trained to lever-press for food (one 0.45-mg food pellet; Bio-Serve, Frenchtown, NJ) on a fixed ratio 1 schedule of reinforcement while food was restricted to 20 g chow/rat per day. Once stable responding was achieved, animals were given ad libitum access to food for the remainder of the experiment. The animals then were surgically prepared with in-dwelling jugular catheters and, after a postoperative recovery period of 7 days, animals were trained to self-administer cocaine on a daily basis in 1-h sessions. Animals were tested in sound-attenuated chambers equipped with a retractable lever. Sessions were initiated by extension of the lever into the operant chamber. Drug infusions were delivered by a Razel (model A, Scientific Instruments, Stanford, CT) syringe pump activated to deliver cocaine through a polyethylene tube attached to the catheter on the animal’s back via a liquid swivel (model 375, Instech Labs, Plymouth Meeting, PA) and a commercially available cannula connector (Plastics One, Roanoke, VA). The daily sessions continued until the total number of cocaine infusions per session stabilized to within ±10% for 3 consecutive days. In all self-administration sessions a lever-press resulted in an i.v. infusion of 0.1 ml solution of cocaine HCl (Sigma) dissolved in saline (2.5 mg/ml) delivered over 4 s. A white cue light above the lever indicated delivery of a cocaine infusion and remained lit for a 20-s timeout, during which responses were recorded but not reinforced.

Surgical Procedure. Rats trained to lever-press for food (see above) were deeply anesthetized under chronic vapor halothane (1.0–1.5%) and implanted with chronic in-dwelling catheters in the jugular vein as described (24). All animals were allowed to recover for a minimum of 7 days before self-administration training. The patency of each catheter was maintained by flushing daily with heparinized (30 units/ml) sterile physiological saline. The integrity of each catheter was tested periodically (on observed erratic performance) by infusion of 0.1 ml of Brevital sodium (1% methohexitol sodium), which resulted in pronounced loss of muscle tone within 3 s of i.v. injection.

mAbs: GNC92H2. The GNC-KLH conjugate (5) was mixed with a Ribi monophosphoryl lipid A (MPL) + synthetic trehalose dicorynomycolate (TDM) adjuvant system (Ribi Immunochem), and the vaccine emulsion was used to immunize mice (strain 129 GIX) that were not older than 3 months of age. The first injection (200 μl) contained the immunocojugate (100 μg based on KLH) and adjuvant (50 μg) reconstituted in PBS. The injection was administered i.p. A second booster injection was given 2 weeks later in a similar fashion. An eye bleed of anesthetized mice 7–10 days later was used to assess the titer. In this case, a titer of >25,000 indicated that no additional i.p. vaccine was necessary. A final tail vein i.v. injection of GNC-KLH (50 μg) in PBS (150 μl) was given 1 month after the final i.p. boost. Three days later, the spleen was fused with a myeloma cell line (X63-Ag8.653) to produce hybridomas according to standard techniques (25, 26) with some modifications developed in our laboratory. The hybridomas were cloned into 96-well plates and screened against GNC-BSA conjugate by ELISA during the cloning process. Each member of a final panel of 19 mAbs then was assessed for binding to cocaine and cocaine metabolites in solution by using equilibrium dialysis (3H-labeled compounds). The mAb GNC92H2 emerged as the clone with the most favorable overall properties of specificity and affinity for cocaine (isotype κγ2a; Kd = 0.24 μM; no cross reactivity with ecgonine or ecgonine methyl ester; benzoylcegonine IC50 = 100 Kd).

Immunization Procedures. Active immunization. Rats were immunized with i.p. injections of a 400-μl bolus of a Ribi adjuvant (MPL-TDM) containing 250 μg of GNC-KLH or KLH in 100 mM PBS, pH 7.4 (5). This initial inoculation was followed by boosts at 21 and 35 days. The last boost was administered without adjuvant, and 7 days later blood was collected through the i.v. catheters and serum samples analyzed by ELISA as described (5).

Passive immunization. Rats received an i.v. bolus injection of 10, 20, 30, and/or 40 mg/kg of the monoclonal IgG GNC92H2 in PBS solution (13 mg/ml) or the control rat IgG (Sigma) in PBS solution (10 mg/ml) 30 min before the onset of the test session.

Self-Administration Reinstatement. The experimental design consisted of six phases: acquisition, extinction, and re-exposures I, II, III, and IV for a total duration of 26 days.

Acquisition. Catheterized animals (n = 20) were trained to self-administer cocaine (0.25 mg/infusion) as described above. After meeting baseline criteria, animals were subjected to an immunization protocol with GNC-KLH (experimental group) or KLH alone (control group), followed by two additional boosters (see above).

Extinction. Immediately after the first immunization, the extinction phase was initiated by replacing cocaine with isotonic saline. Animals were subjected to saline self-administration sessions on a daily basis until lever-pressing behavior resembled extinguished-like values (<5 responses per h) and up to the onset of re-exposure I.

Re-exposure I. Upon extinction and 3 days after the last booster, rats were tested for cocaine self-administration reinstatement by priming with cocaine (0.25 mg/infusion) before session onset as described above. Reinstatement of responding did not result in a drug infusion, because the syringe pump was disconnected postpriming injection. Rats were subjected to such re-exposure sessions for 2 consecutive days (re-exposure days 1 and 2).

Re-exposure II. After the delivery of the priming infusion (0.25 mg/infusion) animals were allowed to self-administer cocaine in the following fashion: one infusion (day 3), two infusions (day 4). Thus, effectively, animals received double (day 3) and triple (day 4) the dose of cocaine.

Re-exposure III. On days 5–7, rats were allowed unlimited access to cocaine self-administration for 1 h.

Re-exposure IV (passive immunization). After a second period of extinction (days 8–15), on day 16 rats were treated with GNC92H2 (GNC-KLH group) or control rat IgG (KLH group) (30 mg/kg i.v.), tested in a 1-h session of unlimited access to cocaine self-administration 30 min after treatment. After behavior was again extinguished (days 17–25) IgG treatment was reversed: the KLH group received GNC92H2 and the GNC-KLH group was treated with the control rat IgG 30 min before the final 1-h session of unlimited access to cocaine self-administration (day 26).

Passive Immunization Dose-Response. Catheterized animals (n = 10) were trained to self-administer cocaine as described above. After meeting baseline criteria, animals were subjected to a period of extinction (see above). Once extinction-like responding was achieved, animals were tested with GNC92H2 or control IgG injections (10, 20, 30, and 40 mg/kg, i.v.) in a Latin square design where each treatment day of a GNC92H2 dose was followed by a treatment day of an equivalent dose of the control IgG, and such 2-day treatment blocks occurred between a period of extinction (7 days).
Between Session Cocaine Self-Administration Dose–Response. Catheterized animals (n = 20) were trained to self-administer cocaine (0.25 mg/infusion) as described above. A preimmunization cocaine dose-effect function (0.015, 0.03, 0.06, 0.25, 0.5 mg/infusion) was generated by using a within-subject Latin square design. The criterion for the start of tests of different unit doses of cocaine was three consecutive self-administration sessions with less than 15% variation in the total number of earned reinforcers. Rats then were immunized with either GNC-KLH (n = 10) or KLH alone (n = 10) as described above. Self-administration sessions were discontinued for the duration of the immunization protocol. Three days after the last booster, animals were again subjected to the within-subject Latin square design.

Statistical Analyses. For the reinstatement study, differences in number of responses per session were analyzed by ANOVA using the immunogen (GNC-KLH or KLH) as the between factor and re-exposure day as the within factor. Data from the “free cocaine” phase were analyzed by comparing them to the corresponding baseline values. All other results were analyzed by using a two-factor ANOVA (with repeated measures on dose and nonrepeated measures on hapten, as appropriate). Individual means comparisons were performed by using Neuman-Keuls a posteriori test after a significant overall effect, as required.

Results
Self-Administration Reinstatement. Four animals were excluded from the study because of failure to acquire cocaine self-administration (n = 1), erratic baseline values (n = 1), or catheter failure (n = 2). The average weight of the animals on completion of the study was 410 ± 38 g. Anticocaine antibody titers were greater than 1:25,000, as previously reported (5). Fig. 1 shows the mean number of responses per session throughout the time continuum of the study. For all rats, baseline criteria were met within 5–8 sessions (Table 1). During the extinction phase, substitution of saline for cocaine resulted in the characteristic initial frustration bursts, followed by a gradual decrease of lever pressing. All animals presented extinction-like levels of responding within 42 days (<5 responses). Reinstatement of self-administration behavior was observed in all vehicle KLH-treated rats throughout the re-exposure phases, and baseline rates of responding values were reinstated (Fig. 1B; Table 1). Conversely, single cocaine priming infusions did not result in reinstatement of animals responding in the GNC-KLH group (Fig. 1A; Table 1). ANOVA revealed significant group differences [F(1,14) = 10.34, P < 0.004; F(1,14) = 19.219, P < 0.001] of operant performance in re-exposure days 1 and 2. On day 3 (two infusions), five of eight rats displayed reinstatement-like behavior [F(1,14) = 0.589, not significant], and on day 4 (three infusions), only three of eight rats increased their response values [F(1,14) = 5.923, P < 0.03]. Free cocaine access produced a 201% increase in the rate of responding in the GNC-KLH group [F(1,14) = 16.779, P < 0.001], whereas control animals performed at baseline levels (Fig. 1A, Table 1). Fig. 2 illustrates the lever-pressing patterns of representative animals from both groups. Passive immunization with GNC92H2 resulted in significant differences in both GNC-KLH and KLH groups when compared with treatment with the control IgG [GNC-KLH: F(1,14) = 18.712, P = 0.003; KLH: F(1,14) = 8.9, P = 0.018] (Fig. 3). The GNC92H2 effect did not differ significantly between groups [F(1,14) = 0.901, not significant]).

Passive Immunization Dose–Response. One animal was excluded from the study because of catheter failure. The average weight of the animals on completion of the study was 327 ± 11 g. Fig. 4 shows the average number of cocaine self-infusions administered post-GNC92H2 or after control IgG treatment (n = 9).

There was a significant main effect of dose [F(3,8) = 24.66, P < 0.0001] and dose × treatment interaction [F(3,24) = 26.086, P < 0.0001]. Analysis of simple main effects revealed a significant difference between GNC92H2- and control IgG-treated cocaine self-administration relapse in rats. Mean response rates per 1-h session are shown during baseline training, extinction (saline substitution), relapse (noncontingent cocaine delivery), and free access to cocaine, under a fixed ratio 1:20 schedule of reinforcement. Animals were treated with either the cocaine conjugate GNC-KLH (A) or the carrier protein KLH (B). * indicate a significant difference between groups during the relapse phase, P < 0.029, ANOVA. † indicate that response rate values for each group were significantly different from their respective baseline values, P < 0.01, ANOVA.

Table 1. Mean number of cocaine infusions (0.25 mg/infusion) ± SEM in 1 hr before (baseline) and after (free cocaine) immunization

<table>
<thead>
<tr>
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<th>GNC-KLH</th>
<th>KLH</th>
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<tbody>
<tr>
<td>Baseline</td>
<td>16.40 ± 1.60</td>
<td>14.62 ± 2.35</td>
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<tr>
<td>Extinction</td>
<td>6.12 ± 1.21</td>
<td>5.93 ± 0.96</td>
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<tr>
<td>Re-exposure</td>
<td></td>
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<tr>
<td>Day 1 (1 prime)</td>
<td>4.30 ± 1.01*</td>
<td>15.00 ± 2.14</td>
</tr>
<tr>
<td>Day 2 (1 prime)</td>
<td>3.90 ± 1.05*</td>
<td>12.20 ± 2.17</td>
</tr>
<tr>
<td>Day 3 (1 prime + 1 infusion)</td>
<td>15.40 ± 4.33</td>
<td>13.20 ± 1.43</td>
</tr>
<tr>
<td>Day 4 (1 prime + 2 infusions)</td>
<td>9.25 ± 3.49*</td>
<td>17.20 ± 1.72</td>
</tr>
<tr>
<td>Free cocaine</td>
<td>29.61 ± 4.31†</td>
<td>14.70 ± 2.63</td>
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Mean number of responses ± SEM in 1 hr before (extinction) and after (re-exposure) immunization.
*Significantly different from control group (KLH).
†Significantly different from baseline.
groups at doses 10, 30, and 40 mg/kg. The increased magnitude of SEM values at the 20 mg/kg GNC92H2 dose denotes response suppression in three of nine animals and a marked intake increase in four of nine animals. Subsequent post hoc analyses indicated a significant dose-dependent increase (dose 10 mg/kg; \( P < 0.05 \)) and decrease (doses 30 and 40 mg/kg; \( P < 0.01 \)) of infusions after GNC92H2 only. Treatment with the control IgG did not affect response rate at any dose [\( F(3,24) 0.232, \) not significant].

**Cocaine Self-Administration Dose–Response.** Four animals were excluded from the study because of failure to acquire cocaine self-administration (\( n = 1 \)) and catheter failure (\( n = 3 \)). The average weight of the animals on completion of the study was 380 ± 26 g. Anticocaine antibody titers were greater than 1:25,000, as previously reported (5). Cocaine produced a characteristic inverted-U-shaped self-administration dose–effect function before immunization, as illustrated in Fig. 5 in the KLH group. Active immunization with GNC-KLH resulted in a clear shift to the right of the entire cocaine dose–effect function measured between sessions, such that lower doses of cocaine were poorly self-administered and higher doses of cocaine were self-administered with a shorter interval between injections [Fig. 5; main effect of cocaine dose, \( F(4,56) 29.175, P < 0.0001 \); immunogen interaction, \( F(4,56) 33.484, P < 0.0001 \)].

**Discussion**

The data presented here are an extension of our previous findings (5) and lend support to the hypothesis that GNC-KLH generates an effective titer of cocaine antibodies that can bind to cocaine before its entrance into the central nervous system. Immunization with GNC-KLH and GNC92H2 significantly inhibited the reinforcing value and, when combined, the intake of self-administered cocaine.

GNC-KLH effectively prevented reinstatement of cocaine self-administration after a single priming cocaine infusion (0.25 mg/0.1 ml per 4 s), and this effect was sustained in 40–60% of the GNC-KLH-treated rats after double and triple i.v. cocaine infusions of the training dose (Fig. 1, Table 1). The dramatic

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**Fig. 2.** Reinforcer delivery record for GNC-KLH (A) and KLH (B) groups on four different phases of the relapse study (rats 13 and 18, respectively). Each horizontal line is a time line representing a 60-min cocaine self-administration session. The horizontal axis denotes time, and each vertical mark represents a single cocaine infusion (0.25 mg/infusion). The experimental phase is shown to the left of each line. The number shown to the right of each time line is the total number of infusions delivered during the session.

**Fig. 3.** Comparison of the effects of pretreatment with GNC92H2 (30 mg/kg) or control IgG (30 mg/kg) on cocaine self-administration between GNC-KLH- and KLH-treated animals in a 1-h session. Horizontal line depicts average baseline value. Values represent means ± SEM in 16 animals (\( n = 8 \)). *, \( P < 0.018 \), ANOVA, significant difference from baseline.

**Fig. 4.** Effects of pretreatment with GNC92H2 or a control IgG on cocaine self-administration in a 1-h session. Horizontal line depicts average baseline value. Values represent means ± SEM in nine animals. *, \( P < 0.05 \), Newman–Keuls test, significant difference from control IgG.

**Fig. 5.** Self-administration dose–effect function for cocaine in animals immunized with GNC-KLH or KLH alone. The unit dose of cocaine was varied between test sessions. Values represent means ± SEM in 16 animals (\( n = 8 \)). *, \( P < 0.018 \), ANOVA.
increases in rate of responding on free cocaine availability in the GNC-KLH group suggest that antibody titers have a limited blockade capacity in the presence of unlimited access to the drug (Fig. 1A, Table 1), resulting in the need for a higher dose of the drug to achieve a reinforcing level of cocaine in the central nervous system. This increase of response rate presumably reflects a decrease in the reinforcing properties of cocaine by virtue of antibody antagonism. In fact, early cocaine self-administration studies established that the rate of responding maintained under simple fixed-ratio schedules increases as the dose is decreased (27, 28). Thus, low to moderate doses of dopamine receptor antagonists increase cocaine self-administration maintained on this schedule in a manner similar to decreasing the unit dose of cocaine. Interestingly, increases in self-administration in the vaccinated group resemble those of rats treated with dopamine antagonists (21, 29) (Fig. 1A). This apparent decrement in dose is consistent with findings from our original report where active immunization with GNC-KLH resulted in a 60–80% decrease in brain cocaine levels (5).

The titer surmountability threshold was successfully elevated by passive immunization with GNC92H2, yielding a full blockade of reinstatement in both GNC-KLH- and KLH-treated groups (Fig. 3). Of note, the antagonistic effects of the GNC92H2 did not appear to be potentiated by the existing titers induced by active immunization. A possible explanation could be that, at the time of this last re-exposure phase, 19–29 days had elapsed since the last booster, hence the GNC-KLH-elicted antibody titers may have not been sufficiently high to produce an additive blockade effect with GNC92H2. Alternatively, a ceiling effect of the GNC92H2 dose used in this study (30 mg/kg) may explain this observation. Indeed, in the passive immunization dose–response study, cocaine self-administration was affected dose-dependently by GNC92H2 in a biphasic fashion, where at the lowest dose (10 mg/kg) antibodies produced an increase of self-infusions, whereas at the largest doses (30, 40 mg/kg) a profound, but similar, suppression in response rate was obtained (Fig. 4).

The rightward shift of the dose–effect function produced by active immunization with GNC-KLH further confirmed and characterized the efficacy of this treatment as a significant blocking agent for the reinforcing properties of cocaine (21, 22) (Fig. 5). The substantial displacement of the ascending limb indicates that titer surmountability occurred at a dose 8-fold greater (0.25 mg/infusion) than the lowest cocaine dose needed to maintain responding (0.03 mg/infusion). This finding is noteworthy on two counts: first, it appears that the antagonistic value of the titers are more than double those of the average dopamine antagonist, which typically are surmounted by 3-fold increases in the training dose of cocaine (23); second, given the rapidity with which the cocaine infusions were delivered (0.1 ml/4 s) the resilience of the titers appears to be robust, especially in view of a report where antibody antagonism of cocaine self-administration was achieved with a much higher drug delivery duration value (0.6 ml/min) than what we report here (7).

The clinical relevance of these studies is notable in light of the high vulnerability to relapse among cocaine abusers and the lack of suitable medication for its treatment (13, 14). Substantial evidence points at two main cocaine-related factors that trigger relapse: environmental stimuli associated with drug use and acute re-exposure to cocaine (30, 31). The therapeutic value of GNC-KLH and GNC92H2 given their mechanism of action directly addresses the latter. Based on the results reported herein, active immunization with GNC-KLH may offer protection against re-exposure to triple the amount of cocaine needed to obtain reinforcing effects. This is of particular interest in light of clinical findings that suggest that the course of relapse episodes may be influenced by the degree to which the substance abuser enjoys the initial use of cocaine after a period of abstinence (32). Therefore, the endogenous, prolonged, antico- 

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