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Fourphit, an Acylating Phencyclidine Derivative, Attenuates Cocaine-Induced Hyperactivity in Rats

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SCHWERI, M. M., B. R. DE COSTA AND K. C. RICE. *Fourphit, an acylating phencyclidine derivative, attenuates cocaine-induced hyperactivity in rats.* PHARMACOL BIOCHEM BEHAV **60**(3) 615–623, 1998.—Fourphit (4-isothiocyanato-1-[1-phenylcyclohexyl]piperidine), an acylating phencyclidine derivative that irreversibly inhibits stimulant binding to the dopamine transporter in vitro (Schweri et al., J. Pharmacol. Exp. Ther. 261:936–942, 1992), was tested in rats for its ability to block the increased locomotor activity caused by cocaine. Administration of Fourphit (20 mg/kg, IV) significantly reduced the hyperactivity caused by challenge with either 15 or 40 mg/kg (−)cocaine·HCl (IP) 24 h later. It also increased the amount of thigmotaxis and decreased the rearing frequency of rats given the higher dose of cocaine. Only negligible effects on behavior were found upon acute administration of the compound by itself, or in response to a saline challenge 24 h later. Activity during the dark cycle immediately following Fourphit administration, however, was moderately depressed. Contrary to the results predicted from its activity in vitro, Fourphit did not inhibit the ex vivo binding of [^{3}H]methylphenidate to stimulant receptors in the striatal tissue of treated rats. These results show that Fourphit can antagonize some behavioral actions of cocaine, but these effects are not likely due to covalent modification of the site on the dopamine transporter recognized by cocaine. © 1998 Elsevier Science Inc.

Cocaine Antagonist Methylphenidate Stimulant Phencyclidine Dopamine transporter
Fourphit (4-isothiocyanato-1-[1-phenylcyclohexyl]piperidine) Rats Striatum Male Female

COCAINE abuse and addiction constitute a major public health problem in the U.S. and worldwide. Presently, there are no therapeutic agents that effectively reduce or reverse the powerful reinforcing properties of cocaine. Information is emerging from preclinical research, however, which suggests that agents that irreversibly (or pseudoirreversibly) inhibit stimulant binding to the dopamine transporter in vitro may have the potential to reduce or reverse the effects of cocaine in vivo. For example, GBR-12909, a member of the class of diphenyl-substituted piperazine derivatives that are reversible but long-lasting inhibitors of stimulant binding (1), has been shown in microdialysis studies to attenuate the elevation of striatal dopamine levels caused by IV injection of cocaine in rats (3). Another example is Metaphit {1-[1-(3-isothiocyanatophenyl)cyclohexyl]-piperidine}, a *m*-isothiocyanato substituted derivative of phencyclidine (14). This compound irreversibly inhibits [^{3}H]stimulant binding to the dopamine transport complex in striatal tissue in vitro (4,19,26), and has been shown to reduce the hyperactivity caused by injection of mice with cocaine, methylphenidate, or mazindol 24 h after pretreatment with the irreversible inhibitor (4,22).

Although intriguing, these findings are not easily interpretable. Metaphit is a long-lasting inhibitor of dopamine uptake in vitro (20,24), while GBR-12909 also blocks transport ex vivo (1,10). In view of the fact that the two compounds share cocaine's putative mechanism of action [i.e., blockade of dopamine transport (25)], they might be expected to act as cocaine agonists rather than antagonists. Indeed, GBR-12909 by itself was shown to increase dopamine levels in microdialysis studies at the same dose at which it decreased the elevation of

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dopamine levels after cocaine challenge (3). In the experiments with Metaphit, no change in stimulant binding or dopamine uptake could be demonstrated in the mice after Metaphit administration, even though it clearly antagonized the locomotor stimulant effects of cocaine (22). These apparent inconsistencies show that there is still much to be learned about the effects of long-acting agents on the dopamine transporter.

The present work was conducted to add to the scant data base in this area by examining the effects of Fourphit (4-isothiocyanato-1-[1-phenylcyclohexyl]piperidine), on the locomotor stimulant effects of cocaine. Fourphit, a positional isomer of Metaphit, contains an isothiocyanato substituent at the 4 position of the piperidine ring of phencyclidine (5), while Metaphit contains the same substituent at the meta position of its aromatic ring (14). Like Metaphit, Fourphit irreversibly inhibits stimulant binding to the dopamine transporter in vitro (21). Testing of this agent thus would provide a third opportunity to empirically determine whether long-lasting inhibition of stimulant binding in vitro is predictive of cocaine antagonism in vivo. If effective, Fourphit could be an attractive alternative to Metaphit as a potential cocaine antagonist. Metaphit had originally been synthesized as an irreversible affinity label for the phencyclidine receptor (14), so it clearly lacks specificity for the dopamine carrier. Fourphit, on the other hand, binds only reversibly to the phencyclidine receptor in vitro(14,21). Furthermore, reports of audiogenic epileptic seizures following administration of Metaphit to rodents raised concerns over its safety (7,8). In contrast, neither a literature search nor preliminary tests in our laboratory revealed any gross behavioral effects associated with Fourphit.

The purpose of the present study was to determine whether Fourphit, like Metaphit, can antagonize cocaine-induced hyperactivity in rats. In addition, ex vivo [^{3}H]methylphenidate binding was determined on the striatal tissue from some of the rats used in the behavioral tests, to determine whether the behavioral effects obtained with Fourphit correlate with its effects on stimulant binding to the dopamine transporter.

METHOD

Chemicals

The hydrochloride salt of Fourphit was prepared as described previously (5). [^{3}H]Methylphenidate (±)-*threo*-[methyl-^{3}H]methylphenidate; specific activity 70.7 Ci/mmol was custom synthesized by Dupont/New England Nuclear, Boston, MA. The hydrochloride salt of the biologically active stereoisomer of cocaine [(−)cocaine·HCl] was obtained from Sigma Chemical Co., St. Louis, MO. All other reagents were obtained from standard commercial sources.

Behavioral Studies

Series I: Effect of Fourphit on the activity of male rats challenged with 15 mg/kg cocaine·HCl 24 h later. Male Sprague–Dawley rats (180–240 g; Harlan Sprague–Dawley, Indianapolis, IN) that had been group housed in the resident animal facilities for at least 1 week under a 12 L:12 D cycle were separated into clear plastic cages (22-1/2″ L × 13″ W × 7″ H) by 1600 h on the day prior to the start of the experiment and allowed to acclimate overnight in the room where the experiments were conducted. On the day of the experiment, the home cage was placed between the bars of an Opto-Varimex-Mini B animal activity monitor (Columbus Instruments, Columbus, OH), which quantitates activity by counting the number of times that the eight infrared beams that are evenly spaced along the length of the apparatus are broken by the movements of a rat. "Ambulatory" activity is registered when a rat interrupts two or more beams in succession, while "nonambulatory" activity is registered if a rat breaks the same beam in succession. "Total" activity is calculated by summing the ambulatory and nonambulatory activity. A partition was placed in the cage to limit the compartment length to 18-1/2″ (the length of the bar holding the infrared beams), and the wire cage lid was replaced with an inverted clear plastic cage of the same size to allow the animal to rear freely. After collecting a 30-min preinjection baseline reading, each rat was then given an intravenous (IV) injection of either Fourphit (20 mg/kg, tail vein IV; $n = 6$) or vehicle (20% ethanol in saline; $n = 6$) in a volume of 1.5 ml/kg and returned to its home cage in the activity monitor. Five minutes later, activity was recorded at 5-min intervals for 30 min to determine whether Fourphit exhibited intrinsic activity compared to controls. The experiments were started between 0900–1030 h or 1300–1400 h during the light cycle. The rats remained in the testing room until 24 h later, when preinjection baseline readings were again recorded for 30 min. Each rat was then challenged with an intraperitoneal (IP) injection of 15 mg/kg cocaine HCl (IP; vehicle was 1 ml saline/kg) and returned to its home cage. After 5 min, activity was recorded at 5-min intervals for the first half hour, then at 10-min intervals for the second half hour.

Series II: Effect of Fourphit on the activity of female rats challenged with 15 mg/kg cocaine·HCl 24 h later. Female Sprague–Dawley rats, 200–225 g were used in this series. The procedure was the same as above, except clear plastic cages with dimensions of 18-1/2″ L × 10-1/2″ W × 7-1/2″ H were used, and the experiments were conducted only in the afternoon. Also, activity after the cocaine challenge was measured at 5-min intervals throughout the 1-h postinjection period.

Series III: Effect of Fourphit on the activity of male rats challenged with 40 mg/kg cocaine·HCl 24 h later. The procedure used was the same as Series II, with the following exceptions and additions. Male rats (207–255 g) were used. Total, ambulatory, and nonambulatory activities were measured at hourly intervals during the dark cycle of both night 1 (acclimatization period) and night 2 (after injection with Fourphit or vehicle, but before cocaine challenge). All Fourphit or Fourphit vehicle injections were given between 1300–1500 h. Approximately 24 h after the Fourphit or vehicle injection, each rat was challenged with 40 mg/kg cocaine HCl in saline (IP, 1 ml/kg) and returned to its home cage, where its activity was monitored at 5 min intervals for 95 min.

The behavior of each rat was also videotaped following cocaine administration for subsequent visual analysis. From the videotape, raters blinded to the treatment scored the following measures: Rearing frequency: the total number of times each rat reared was summed over a 5-min interval for 90 min after cocaine injection. Rearing was scored when both forepaws left the floor of the cage simultaneously. Thigmotaxis: the number of times each rat completely circled the perimeter of the cage in one direction, without interruption, was summed over each consecutive 5-min interval for 90 min after cocaine injection. Stereotypy: At 5-min intervals for 90 min after cocaine challenge, the rat behavior was observed for 1 min and scored according to the following scale: 1) asleep; 2) awake, but resting; 3) normal in-place activity, like grooming or sniffing at a normal pace; 4) normal active behavior (moving around cage at a relaxed pace and sniffing here and there);

5) normal locomotor activity accompanied by constant sniffing; 6) pure hyperactive locomotor activity (not accompanied by any other atypical behavior); 7) hyperactive locomotor behavior accompanied by brief (<3 s) periods of stereotypical behavior (e.g., sniffing, rearing, grooming, head weaving, or listing to one side); 8) (a) hyperactive locomotor behavior accompanied by intermediate (4–19 s) periods of stereotypical behavior, or (b) a sequence of stereotyped behaviors lasting the whole observation period, linked together by brief locomotor activity of less than 3 s; 9) random hyperactive locomotor behavior interspersed with stereotypical behavior in the same location for longer than 20 s at a time; (10) (a) constant moving around the cage in one direction for the entire observation period (can be interrupted by stops for stereotypy of 10 s or less), or (b) one continuous stereotypical behavior in one spot for the whole observation period; 11) repetitive gnawing, biting, or licking lasting over 20 s continuously; 12) seizures or "popcorn-like" bouncing. The scores were summed over the 90-min period.

Series IV: Effect of Fourphit on the activity of male rats challenged with saline 24 h later. Same as Series I, except all rats were challenged with saline instead of cocaine·HCl.

Ex Vivo [^{3}H]Methylphenidate Binding

At the end of selected experiments, each rat was sacrificed and its striatal tissue quickly removed and assayed for [^{3}H]methylphenidate binding as described previously (21), except that in most instances the tissue suspension was frozen prior to assay. Briefly, the tissue was homogenized in 2 ml 0.32 M sucrose. A P_2 fraction was prepared by differential centrifugation and resuspended in 5 ml Tris assay buffer (50 mm Tris-Cl, 100 mM NaCl, pH 7.4 at RT). If the sample was to be frozen, it was placed in dry ice until solid and stored at −80°C. When the experimental series was completed, the samples were thawed, centrifuged at 20,000 × *g* for 5 min, and resuspended in 3.4 ml Tris assay buffer. For fresh (unfrozen) samples, the P_2 fraction was suspended in 4 ml Tris assay buffer and used without further centrifugation. Next, 400 μl of the tissue suspension was combined with 500 μl of assay buffer, 50 μl water or 200 μM amfonelic acid (to define nonspecific binding), and 50 μl (±)-[*threo-methyl*-^{3}H]-methylphenidate (70.7 Ci/mmol). The final concentration of [^{3}H]methylphenidate in these samples was 9–11 nM. After 30 min incubation at 0°C, the samples were filtered under vacuum through GF/B filters held in a Millipore filtration manifold, and washed twice with 5-ml aliquots of assay buffer. The filters were then shaken with Beckman Ready-Protein scintillation fluid and counted in a liquid scintillation counter. The specific binding obtained in this manner was normallized to protein content of the tissue, determined by the Miller modification of the Lowry method (12).

Statistical Analysis

Results are expressed as mean ± SEM. The locomotor activity data were analyzed by two- or three-way ANOVA with repeated measures (treatment × time after drug injection × time of day), as appropriate. The data were subjected to log or square-root transformations to reduce variance, if necessary. If significance was obtained, individual comparisons between Fourphit and vehicle-treated rats were made with Student's *t*-test. The thigmotaxis, rearing, and [^{3}H]methylphenidate binding data were analysed using Student's *t*-test.

RESULTS

Effects of Fourphit on Cocaine-Induced Behaviors

Series I: Effect on the activity of male rats challenged with 15 mg/kg cocaine·HCl 24 h later. Treatment with Fourphit (20 mg/kg, IV) attenuated the locomotor response of male rats to 15 mg/kg cocaine·HCl (IP) injected 24 h later, compared to the activity of controls (Fig. 1A–C). Controls were treated with vehicle in place of Fourphit. After collapsing the activity counts into 20-min segments, a three-way ANOVA was performed on the data (activity during a 20-min time interval × treatment × time of day); the latter factor was included after initial inspection of the data suggested that the rats studied in the afternoon were more active than those treated in the morning. A significant time of day effect was indeed observed for total and ambulatory activity, $F(1, 8) = 12.50$ and 14.28, $p = 0.008$ and 0.005, respectively. A significant time interval × treatment interaction was seen for total, ambulatory, and nonambulatory activity, $F(2, 16) = 5.61$, 5.41, and 4.28; $p = 0.01$, 0.02, and 0.03, respectively. Locomotor activity in the first 40 min after challenge with cocaine·HCl was significantly reduced by 56% (total), 62% (ambulatory), and 36% (nonambulatory) in Fourphit-treated rats compared to controls (one-tailed paired *t*-test, $p = 0.04$, 0.05, and 0.04, respectively).

Series II: Effect on the activity of female rats challenged with 15 mg/kg cocaine·HCl 24 h later. Experiments similar to those described above were conducted using female rats, except all of the experiments were conducted in the afternoon to eliminate the influence of time of day on the results. The pattern of the total, ambulatory, and nonambulatory activity obtained for the vehicle ($n = 6$)- and Fourphit ($n = 5$)-treated rats over a 1-h period beginning 5 min after injection with cocaine is shown in Fig. 1D–F. Analysis of the cumulative activity data recorded over the 1-h period showed that the Four-

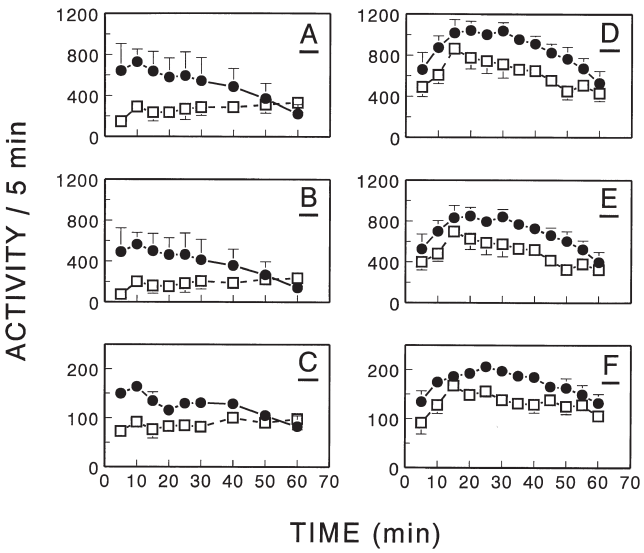

FIG. 1. Effect of Fourphit on activity caused by a low dose (15 mg/kg, IP) of (−)cocaine HCl in rats. Activity counts were recorded over a 1-h period beginning 5 min after cocaine administration to rats that had been treated 24 h previously with either 20 mg/kg (IV) Fourphit (□) or vehicle (●). Six rats were in each treatment group. (A–C) Total, ambulatory, and nonambulatory activity, respectively, in male rats (Series I). (D–F) Total, ambulatory, and nonambulatory activity, respectively, in female rats (Series II). See the Method section for details and the Results section for statistical analysis.

phit-treated rats exhibited significantly less total, ambulatory, and nonambulatory activity in response to cocaine than the vehicle-treated control rats. The results obtained by one-tailed independent *t*-test are as follows: 1) total activity counts/h: vehicle = 10,290 ± 738, Fourphit = 7,446 ± 451 (27.6% decrease), $p = 0.006$; 2) Ambulatory activity counts/h: vehicle = 8,217 ± 644, Fourphit = 5,858 ± 397 (28.7% decrease), $p = 0.008$; 3) nonambulatory activity counts/h: vehicle = 2,073 ± 104, Fourphit = 1,588 ± 90 (23.4% decrease), $p = 0.004$.

Series III: Effect on the activity of male rats challenged with 40 mg/kg cocaine·HCl 24 h later. In an attempt to increase the level of activity in the controls and lessen the variability of the locomotor response to cocaine in both the controls and Fourphit-treated rats, a series of experiments was conducted under the same conditions described above, except a higher dose of cocaine·HCl (40 mg/kg, IP) was utilized. The locomotor activity recorded at 5 min intervals for 95 min after cocaine challenge of vehicle ($n = 7$)- and Fourphit ($n = 10$)-treated rats is shown in Fig. 2. Square root transformations to decrease variability were conducted on the total, ambulatory, and nonambulatory activity counts, after first collapsing the data into 3 consecutive 25-min time segments beginning at 15 min and ending at 90 min after cocaine injection. Two-way (time × treatment) ANOVA of the transformed data showed significant time × treatment interactions for total, $F(2, 30) = 3.22$, $p < 0.05$, and ambulatory, $F(2, 30) = 4.39$, $p < 0.02$, activity, but not for nonambulatory activity, $F(2, 30) = 0.33$, NS. Fourphit-treated rats showed a 34% decrease in total activity (1963 ± 251 vs. 2963 ± 471, $p < 0.03$) and a 43% decrease in ambulatory activity (1268 ± 257 vs. 2233 ± 441; $p < 0.03$) during the 15–40 min period after challenge with cocaine (segment 1 in Fig. 2), but not at the later time periods (independent *t*-test, one-tailed).

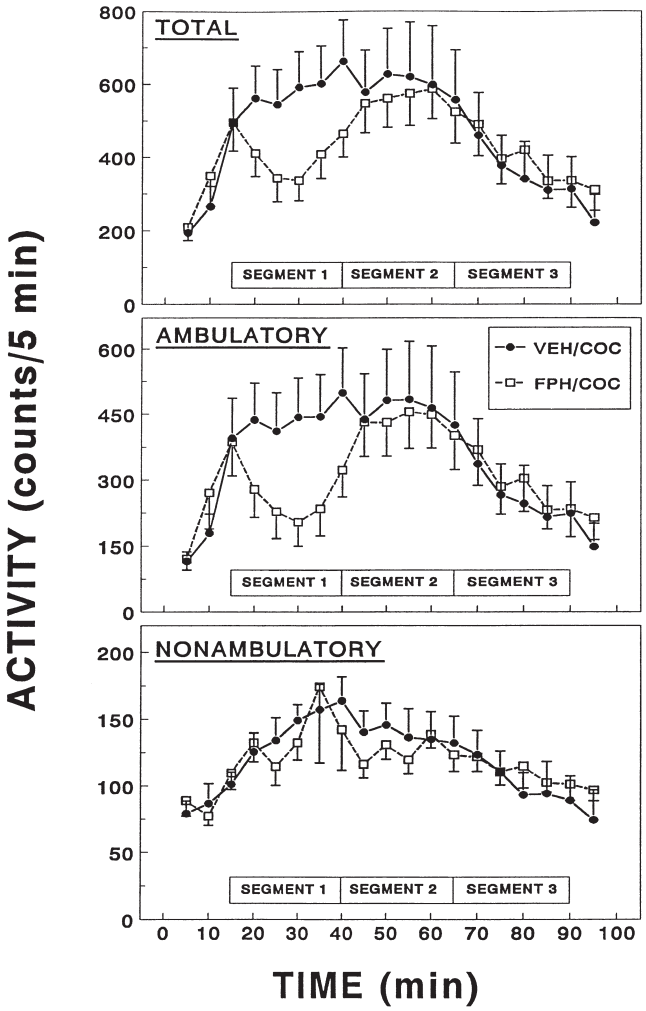

FIG. 2. Effect of Fourphit on activity caused by a high dose (40 mg/kg, IP) of (−)cocaine HCl in male rats. Total, ambulatory, and nonambulatory activity counts recorded over a 95-min period beginning 5 min after administration of cocaine to male rats that had been treated 24 h previously with either 20 mg/kg (IV) Fourphit (□) or vehicle (●). Seven rats were in the vehicle-treated group, and 10 rats were in the Fourphit-treated group. See the Method section for details (Series III). The three segments denote the three 25-min time periods over which activity counts were summed prior to statistical analysis; see the Results section for details.

Although the nonambulatory activity (generally considered a measure of stereotypy) of the Fourphit-treated rats did not differ significantly from that of the controls, direct observation of the animals nevertheless gave the impression that treatment with Fourphit altered cocaine-induced stereotypy. Stereotypic behavior was, therefore, rated on a 12-point scale from the videotapes made of each session by raters blinded to the treatment given each rat (see the Method section). The scores, which represent overall stereotypy for the 90-min period following cocaine administration, did not differ significantly between the vehicle (126 ± 12) and the Fourphit (141 ± 5)-treated groups.

Subsequent analysis, however, revealed that Fourphit signficantly influenced individual components of cocaine-induced stereotypy. Thus, when rearing and thigmotaxis (circling the perimeter of the cage; see the Method section for complete description), two behaviors that were judged from visual inspection to be the most susceptible to Fourphit treatment, were examined individually, significant effects emerged. The number of times each animal reared was recorded at 5-min intervals over the 90 min period following cocaine injection (Fig. 3A). The Fourphit-treated rats engaged in 46% fewer rearings than vehicle-treated controls over the 90-min observation period (205 ± 56 vs. 381± 83, respectively; $p \leq 0.04$, one-tailed independent *t*-test). On the other hand, Fourphit-treated rats exhibited significantly more thigmotactic behaviors than the vehicle-treated rats following a cocaine challenge (Fig. 3B). The Fourphit-treated rats circled their cages 57 ± 15 times over the 90-min period, compared to only 16 ± 9 times for the vehicle-treated animals ($p \leq 0.03$, one-tailed independent *t*-test).

Intrinsic Effects of Fourphit on Behavior

Series IV: Effect on the activity of male rats challenged with saline 24 h later. To determine whether Fourphit alone affected the locomotor behavior of the rats in the experimental paradigms described above, a series of experiments was conducted in which male rats were subjected to the identical experimental conditions described in Series I above, except saline was injected in place of cocaine 24 h after treatment with Fourphit or vehicle. Fourphit had no significant effect on the ensuing activity of the rats under these conditions; for instance, the total activity of the rats treated with vehicle was 382 ± 85 counts/h after saline injection, while the Fourphit-

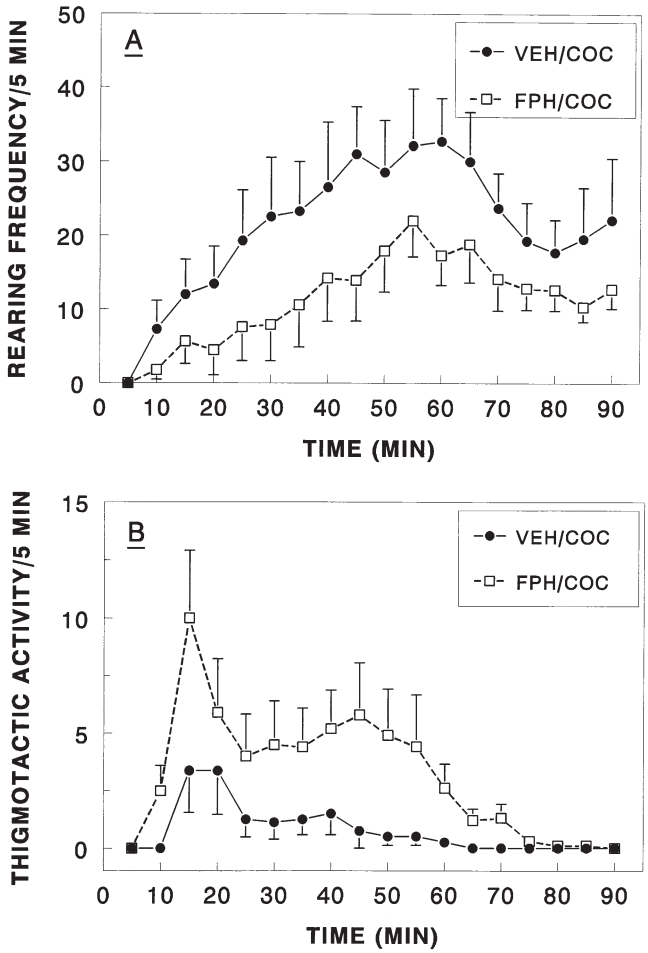

FIG. 3. Effect of Fourphit on selected cocaine-induced stereotyped behaviors. Male rats pretreated with either vehicle (●) or Fourphit (□) 24 h previously were injected with (−)cocaine·HCl (40 mg/kg,IP) and videotaped for 90 min thereafter. Seven rats were in the vehicle-treated group, and 10 rats were in the Fourphit-treated group. See the Method section for details (Series III). (A) Rearing frequency was recorded at 5-min intervals from the videotape. After challenge with cocaine, Fourphit-treated rats engaged in 46% fewer rearings than vehicle-treated controls ($p < 0.04$, one-tailed independent t-test) over the 90-min observation period. (B) Thigmotactic activity (defined as the number of times an animal completely circled the perimeter of the cage in one direction, without interruption) was measured over 5-min intervals. Rats pretreated with Fourphit engaged in 356% more thigmotactic behaviors than the vehicle-treated controls during the 90-min recording period (56.9 vs. 16.0 complete circlings, respectively; $p < 0.03$, one-tailed independent t-test).

treated rats exhibited 442 ± 122 counts/h. Similar results were obtained for ambulatory and nonambulatory activity (data not shown). As expected, these counts were quite low compared to the activity recorded in similarly treated rats following a cocaine injection; for comparison, the rats injected with 15 mg/kg cocaine·HCl in Series I achieved total activity counts of 5,890 ± 1906/h and 3,341 ± 583/h, respectively.

Acute effect of Fourphit on locomotor activity. Fourphit caused a slight, but significant, elevation of locomotor activity immediately after injection. The ambulatory activity of the rats from Series III in the 35-min period after treatment with Fourphit or vehicle is shown in Fig. 4A. Discounting the first 5-min period after the rats were returned to their cages following injection, the average ambulatory activity counts recorded for the Fourphit-treated rats in the remaining 30 min was 170 ± 27 ($n = 9$), compared to 56 ± 17 ($n = 7$) for the vehicle-treated controls; $p \leq 0.005$, two-tailed independent t-test. Similar small, but statistically significant, increases in the total and nonambulatory activity were also observed (data not shown). This experiment was repeated twice more with similar results. As with the results described in the previous section, the increase in activity was negligible compared with that seen following the administration of cocaine.

Effect of Fourphit on activity during the dark cycle. In view of the fact that rodents are most active at night, it was of interest to determine whether the slight activating effect observed immediately after Fourphit administration would be even more pronounced during the following dark cycle. The activity of the Fourphit- and vehicle-treated rats from Series III was recorded in 1-h segments overnight; the ambulatory activity is presented in Fig. 4B. Instead of the predicted effect, Fourphit was found to influence the dark cycle behavior of the rats in a complex manner, ultimately decreasing it. As shown in Fig. 4B, Fourphit appeared to attenuate the activity of the rats during their peak activity periods, but have little effect on their behavior during periods of low activity. Ambulatory activity in the 11-h period beginning one-half hour after the lights were turned off and ending one-half hour before the lights were turned on was reduced by 26% in Fourphit-treated rats compared to controls (5856 ± 377 vs. 7862 ± 336, respectively; $p \leq 0.001$, one-tailed independent t-test). In contrast, the activity of the rats recorded during the same period the preceding night (prior to any treatment) was not significantly different. Similar behavioral patterns were observed for the total and nonambulatory activity (data not shown).

Effect of Fourphit on [^{3}H]Methylphenidate Binding

When the final set of behavioral measurements was completed for a rat, it was sacrificed within approximately 1 h or less, and its striatal tissue was removed and frozen. Upon conclusion of an experimental series (except for Series III, which was not studied), [^{3}H]methylphenidate binding was determined on all of the samples from a particular series within the same assay, to minimize assay-to-assay variation. If Fourphit irreversibly acylates the stimulant binding site on the dopamine transporter, [^{3}H]methylphenidate binding was expected to be lower in the striatal tissue from the Fourphit-treated rats, compared to the vehicle-treated controls. No difference was found, however, between [^{3}H]methylphenidate binding in the two treatment classes (Table 1)

To examine the possibilities that 1) the expected decrease in stimulant binding could only be seen in fresh tissue, and 2) the effect would be more evident if the binding studies were conducted closer to the time of Fourphit administration, an additional experiment ("Series V") was conducted in which [^{3}H]methylphenidate binding was determined in fresh striatal tissue taken from rats sacrificed exactly 1 h after treatment with Fourphit or vehicle. Although the specific binding was higher in both treatment groups when fresh tissue was used, no difference was found in [^{3}H]methylphenidate binding between the two groups (Table 1).

DISCUSSION

The rationale for proposing that Fourphit would antagonize the behavioral actions of cocaine was based on reports describing the antagonistic efficacy of a related compound,

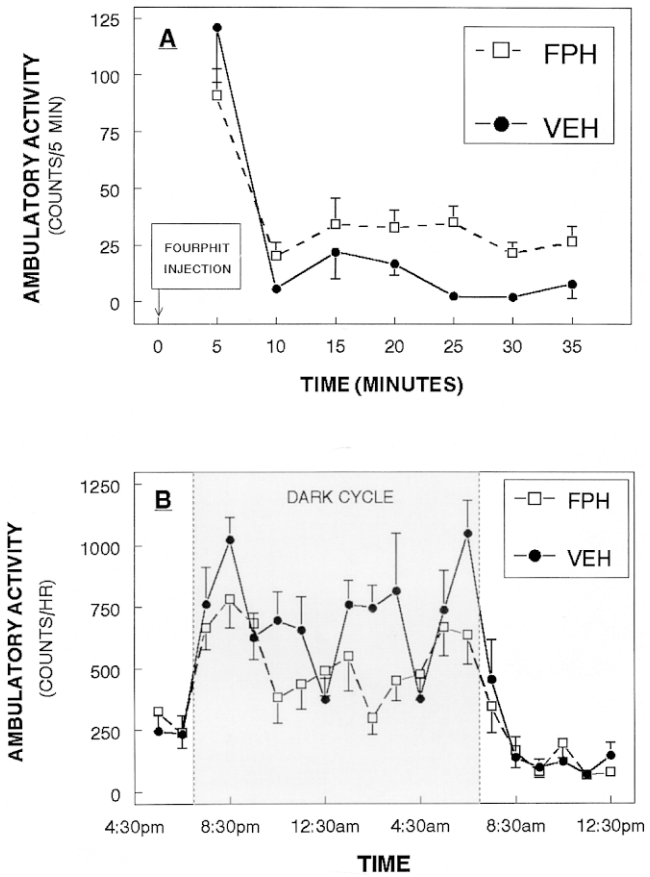

FIG. 4. Intrinsic effect of Fourphit on ambulatory activity acutely (A) and overnight (B). Data shown are from the Series III experiments. (A) Activity counts were recorded at 5-min intervals for 35 min after injection of male rats with 20 mg/kg (IV) Fourphit (□) or vehicle (●). The ambulatory activity measured between 5 and 35 min after injection was higher in Fourphit-treated rats compared to vehicle-treated controls (170 ± 27 [$n = 9$] and 56 ± 17 [$n = 7$] counts per 30 min, respectively; $p < 0.005$, two-tailed independent t-test). Similar results were obtained for total and nonambulatory activity. (B) Activity counts were recorded at hourly intervals for 20 h spanning the first dark cycle (1900–0700 h) after treatment of rats with Fourphit (20 mg/kg, IV) or vehicle. Ambulatory activity in the 11-h period from 1930–0630 h was reduced by 26% in Fourphit-treated rats ($n = 8$) compared to vehicle-treated controls ($n = 7$), (5860 ± 380 vs. 7860 ± 340, respectively; $p < 0.001$, one-tailed independent t-test). Similar results were obtained for total and nonambulatory activity. No differences were observed in the locomotor activity of the same rats the night before treatment with Fourphit or vehicle (data not shown).

Metaphit (4,22), along with our findings that Fourphit, like Metaphit, irreversibly inhibits stimulant binding in vitro (21). This led to the empirically derived notion that long-acting inhibition of stimulant binding in vitro may be a means of identifying compounds that will antagonize the stimulant properties of cocaine in vivo. Subsequent reports on the pseudo-irreversible dopamine uptake inhibitor, GBR 12909 (18), also supported this theory. GBR 12909 was found to attenuate the increase in extracellular dopamine levels in the nucleus accumbens caused by IV injection of cocaine (3). Although the behavioral studies with Metaphit were conducted in mice, the similarities between the results obtained with Fourphit and Metaphit suggest that the two isomers may share the same (as yet undefined) mechanism of action. Both compounds, when administered 24 h prior to cocaine challenge, decreased the locomotor behavior caused by the subsequent IP injection of cocaine (present work; (4,22)). When challenged with a low dose of cocaine·HCl (15 mg/kg), both male and female Fourphit-treated rats exhibited a simple pattern of reduced locomotor activity that persisted until the cocaine-induced hyperactivity began to wane (Fig. 1).

Male rats that received the same dose of Fourphit as the rats described above, but a higher dose of cocaine·HCl (40 mg/kg), exhibited a unique triphasic pattern of total and ambulatory activity after cocaine administration (Fig. 2). Their activity was reduced transiently between 15 and 40 min after stimulant injection, but then rose to the same levels as the vehicle-pretreated cocaine-injected controls. The mechanism underlying this novel behavioral sequence is unclear. It is possible that this transient attenuation of cocaine-induced hyperactivity by Fourphit reflects a selective effect on one of the dopamine storage compartments. Unfortunately, the available data on Metaphit is integrated over a 30-min time period (4,22), thereby obviating the possibility of a direct comparison of its effect on activity with the influence of Fourphit on the temporal pattern of cocaine-induced behaviors, which was measured at 5-min intervals.

Although both Metaphit (4,19,26) and Fourphit (21) irreversibly inhibit stimulant binding when added to tissue preparations in vitro, neither compound has been shown to reduce stimulant binding in tissue removed from rodents injected with the respective agent [Table 1 and (22,26)]. Despite the utilization of a variety of approaches in the ex vivo studies reported here, we were unable to demonstrate the expected reduction in [^{3}H]methylphenidate binding in the striatal tissue of rats who had been treated 26 h previously with Fourphit, compared with that from vehicle-treated controls (Series I, II, and IV; Table 1). Two possibilities were considered as potential explanations for this. First, the behavioral effects of Fourphit measured a day after its administration may have been caused, not by the continuous inhibition of the stimulant binding site by Fourphit, but by functional alterations of dopaminergic pathways set into motion by the interaction of Fourphit with the dopamine transporter soon after it was injected; in this scenario, the functional alterations persist, but homeostatic mechanisms restore the [^{3}H]methylphenidate binding back to normal during the intervening 26 h. To address this possibility, [^{3}H]methylphenidate binding was determined in unfrozen striatal tissue removed from rats treated with either vehicle or Fourphit 1 h before sacrifice. No reduction in binding was observed in the tissue from the Fourphit-treated rats even under these conditions (Table 1, Series V). These results suggest that acute interaction of Fourphit with the stimulant binding site is not the triggering mechanism that leads to functional changes that manifest as inhibition of cocaine-induced behavioral activation 24 h later. They are consonant with the results of Sershen et al. (22), who detected no change in the ex vivo binding of [^{3}H]cocaine 2 or 24 h after treatment with Metaphit, nor in the in vivo binding of [^{3}H]GBR 12935 24 h after treatment with the compound. In that study, a significant increase in the brain concentration of homovanillic acid, a dopamine metabolite, was observed in the Metaphit-treated mice, however, indicating that some long-lasting alteration of the dopamine system had nonetheless occurred.

The possibility was also entertained that the use of frozen tissue may have obscured the results. The binding studies from Series I, II, and IV were conducted on tissue collected

TABLE 1

EFFECT OF FOURPHIT ON EX VIVO SPECIFIC BINDING OF [^{3}H]METHYLPHENIDATE

Series	Sex	Challenged With	Interval to Sacrifice†	Tissue Status	Specific Binding* (CPMs/mg protein)	
					Vehicle	Fourphit
I	Male	Cocaine·HCI (15 mg/kg)	26	Frozen	43,200 ± 2,000 (6)	51,400 ± 5,400 (6)
II	Female	Cocaine·HCI (15 mg/kg)	26	Frozen	53,800 ± 3,000 (6)	51,200 ± 3,600 (5)
IV	Male	Saline	26	Frozen	39,900 ± 3,000 (6)	36,900 ± 2,900 (6)
V	Male	—	1.0	Fresh	70,880 ± 5,400 (5)	77,400 ± 3,200 (5)

Rats were given IV injections of vehicle (20% ethanol in saline) or Fourphit (20 mg/kg) prior to challenge with cocaine or saline, as dictated by the conditions of each experimental series (see the Method section). Striatal tissue was removed and stored as described under "Tissue Status" prior to assay of [^{3}H]methylphenidate binding.

*Values reported as mean ± SEM (n).

†Interval between administration of vehicle or Fourphit and sacrifice.

from the same rats that were used for the behavioral studies, to measure behavior and stimulant binding in the same animal. Due to the lengthy procedures involved and the limited number of video and activity monitors available, the logistics of these experiments did not allow for the collection of the behavioral and binding data on all of the subjects within the same day. Accordingly, behavioral data were gathered from equal numbers of vehicle- and Fourphit-treated rats each day. A P_2 fraction was then prepared from the striatal tissue of each rat, suspended in Tris-Cl buffer, and frozen until the series was completed; then all of the samples from a series were analyzed for [^{3}H]methylphenidate binding in one assay. In a control study, we found a 35.8% loss of binding in frozen tissue compared to fresh tissue, when samples prepared under the two conditions were assayed within the same experiment. After all of the experimental series had been completed, reports emerged in the literature that freezing alters the K_D and/or B_{max} of [^{3}H]WIN 35,428 binding (9,15). Although our control study had shown a reduction in binding after freezing, we thought it likely that this reduction would affect the Fourphit- and vehicle-treated samples equally, and that inhibition by Fourphit, if it occurred, would manifest as a further decrease in binding compared with that of vehicle-treated rats. Indeed, Rothman et al. had successfully demonstrated a pseudoirreversible inhibition of stimulant binding by GBR-12909 utilizing a similar procedure (18). Comparison of the binding data for the fresh tissue (Series V) with frozen tissue (Series I, II, and IV) in Table 1 shows that binding to the frozen tissue samples did indeed decrease in the range predicted by our control study, but no further loss was seen in the Fourphit-treated rats. Good reproducibility was obtained under both conditions: the SEM ranged from 4.1–7.6% and 4.6–10.5% of the mean for the fresh and frozen tissue, respectively; no systematic correlations of binding with length of freezer storage were observed. In short, freezing appeared to reproducibly lower [^{3}H]methylphenidate binding, but did not appear to introduce artifacts that would obscure the detection of a Fourphit-induced decrease in binding. Because our experimental paradigm was constructed to measure locomotor activity and binding within the same rat, sufficient striatal tissue was not available to conduct individual Scatchard analyses. Binding was measured at only one concentration of [^{3}H]methylphenidate in these experiments; thus, effects on the K_D and B_{max} could not be determined.

The most straightforward interpretation of the absence of a Fourphit-induced change in [^{3}H]methylphenidate binding in conjunction with a reduction in the behavioral response to cocaine is that, in vivo, Fourphit (or its active metabolite) functions as a physiological, rather than a pharmacological antagonist. Physiological antagonists block the effects of a drug indirectly by enhancing the activity of other opposing systems, rather than by a direct antagonism of the receptor recognized by the drug (6). Although merely speculative at this time, integration of several divergent lines of research points to the possibility that Fourphit and Metaphit could serve as physiological antagonists of cocaine by interacting with calcium channels located on dopamine nerve terminals. Both of these acylating phencyclidine derivatives have been shown to irreversibly increase the affinity of the dihydropyridine calcium channel antagonist [^{3}H]nitrendipine for its receptor in mouse brain in vitro (5). They have also been shown to attenuate K^+stimulated $^{45}Ca^{2+}$ uptake into nerve terminal preparations in vitro (16). The dihydropyridine calcium channel antagonists block cocaine-induced hyperactivity and dopamine release, but have reduced or no efficacy against amphetamine-induced hyperactivity and dopamine release (2,13,17). Similarly, both Metaphit and Fourphit block cocaine-induced hyperactivity, but do not inhibit the hyperactivity caused by amphetamine [(22); unpublished data on Fourphit from MS, manuscript in preparation]. Cocaine-mediated increases in synaptic dopamine require calcium, while amphetamine releases dopamine in a calcium-independent manner [see references in (13)]. Taken together, these results suggest that Fourphit and Metaphit could act as physiological antagonists of cocaine by affecting calcium channel function in vivo.

When challenged with the higher dose of cocaine·HCl (40 mg/kg) after 24 h, the Fourphit-treated rats showed a significantly lower incidence of rearing (Fig. 3A) and a higher degree of thigmotaxis (Fig. 3B) than the vehicle-treated controls. Neither behavior was reflected in the measures of locomotor activity obtained from the animal activity monitors; rather, visual inspection of the videotapes of the cocaine-challenge sessions was required for quantification (see the Method section). It is unclear why the reduced rearing was not recorded as a decrease in nonambulatory activity counts (Fig. 2); it is possible that other ongoing activities (such as sniffing and random head movements), which occurred simultaneously, may have obscured the results. Nevertheless, the decrease in rearing is consistent with the reduction in cocaine-stimulated total and ambulatory activities seen after Fourphit administration. The increase in thigmotactic activity, however, seems inconsistent with the behavior expected from a

cocaine antagonist. This trait often emerges when a rodent is placed in a novel environment, and is generally considered to be a measure of emotionality, particularly anxiety (23). Phencyclidine, the parent compound of Fourphit, has itself been shown to decrease rearing and increase thigmotaxis (11). Many other drugs, including dopamine uptake blockers like GBR 12783, amphetamine, and cocaine, have also been reported to increase this wall-hugging behavior in a novel environment (23). With regard to this specific trait, then, Fourphit appears to potentiate, rather than antagonize, cocaine's effects.

Fourphit itself has a negligible effect on activity when behavior is observed acutely following its injection or in response to a saline injection 24 h later. Although the locomotor activity of the Fourphit-treated rats was statistically greater than that of the vehicle-injected controls in the 30-min period after treatment (Fig. 4A), the net activity was trivial (e.g., 170 ambulatory activity counts) compared to the counts normally associated with stimulant-induced behavior. For example, control rats from the same series registered 1,980 counts of ambulatory activity in the same period following treatment with 40 mg/kg cocaine·HCl.

In view of the unremarkable behavioral effects of Fourphit described above, it was surprising to find that it significantly decreased activity during the dark cycle, when the rats are most active. Inspection of the overnight record reveals a complex pattern of activity, rather than a mere reduction in activity across all time points (Fig. 4B). This suggests that Fourphit may selectively impact specific behaviors, but does not exert a generalized debilitating effect on the rats. This impression was borne out in our experience in working with the rats: the Fourphit-treated rats did not appear sick, nor did their behavior during handling differ from the control group.

Although the data reported here suggest that Fourphit is certainly not the end point in the search for the ideal cocaine antagonist, it merits consideration as a lead compound that might be further modified to eliminate its remaining undesirable properties but retain its promising aspects. Features that presage its usefulness as an antagonist are 1) it significantly attenuates the stimulant effects of cocaine, as reflected in its reduction of both the elevated locomotor activity (Figs. 1 and 2) and increased rearing (Fig. 3A), which follow cocaine administration; 2) it does not require administration immediately prior to cocaine use to be effective; and 3) it possesses little or no cocaine-like intrinsic activity (Fig. 4A), suggesting that it and drugs like it may have low abuse potential. On the other hand, characteristics that make Fourphit undesirable in its present form are 1) it affects normally occurring behavior in the absence of cocaine (e.g., it alters nocturnal behavior, Fig. 4B); 2) it increases thigmotaxis after cocaine challenge (Fig. 3B), which may translate into increased anxiety levels in human cocaine users; and 3) it offers only delayed and transient protection against higher cocaine doses (Fig. 2), suggesting that it may not prove useful in crack smokers, where it is most needed. Hopefully, the information provided here will ultimately contribute to the development of more satisfactory agents for the treatment of cocaine abuse and addiction.

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REFERENCES

1. Andersen, P. H.: The dopamine uptake inhibitor GBR 12909: Selectivity and molecular mechanism of action. Eur. J. Pharmacol. 166:493–504; 1989.
2. Ansah, T.-A.; Wade, L. H.; Shockley, D. C.: Effects of calcium channel entry blockers on cocaine and amphetamine-induced motor activities and toxicities. Life Sci. 53:1947–1956; 1993.
3. Baumann, M. H.; Char, G. U.; De Costa, B. R.; Rice, K. C.; Rothman, R. B.: GBR12909 attenuates cocaine-induced activation of mesolimbic dopamine neurons in the rat. J. Pharmacol. Exp. Ther. 271:1216–1222; 1994.
4. Berger, P.; Jacobsen, A. E.; Rice, K. C.; Lessor, R. A.; Reith, M. E. A.: Metaphit, a receptor acylator, inactivates cocaine binding sites in striatum and antagonizes cocaine-induced locomotor stimulation in rodents. Neuropharmacology 25:931–933; 1986.
5. Bolger, G. T.; Rafferty, M. F.; Weissman, B. A.; Rice, K. C.; Skolnick, P.: Acylating phencyclidines irreversibly enhance brain calcium antagonist binding. Pharmacol. Biochem. Behav. 25:51–57; 1986.
6. Bourne, H. R.: Drug receptors and pharmacodynamics. In: Katzung, B. G., ed. Basic and clinical pharmacology. Stamford: Appleton & Lange; 1998:9–33.
7. Debler, E. A.; Lipovac, M. N.; Lajtha, A.; Zlokovic, B. V.; Dunlop, D. S.; Jacobson, A. E.; Rice, K. C.; De Costa, B.; Reith, M. E. A.: Metaphit-induced audiogenic seizures in mice: I. Pharmacologic characterization. Epilepsia 34:201–210; 1993.
8. Debler, E. A.; Lipovac, M. N.; Lajtha, A.; Zlokovic, B. V.; Jacobsen, A. E.; Rice, K. C.; Reith, M. E. A.: Metaphit, an isothiocyanate analog of PCP, induces audiogenic seizures in mice. Eur. J. Pharmacol. 165:155–159; 1989.
9. Kirifides, A. L.; Harvey, J. A.; Aloyo, V. J.: The low affinity binding site for the cocaine analog, WIN 35,428 is an artifact of freezing caudate tissue. Life Sci. 50:PL139–PL142; 1992.
10. Koff, J. M.; Shuster, L.; Miller, L. G.: Chronic cocaine administration is associated with behavioral sensitization and time-dependent changes in striatal dopamine transporter binding. J. Pharmacol. Exp. Ther. 268:277–282; 1994.
11. Lehman-Masten, V. D.; Geyer, M. A.: Spatial and temporal patterning distinguishes the locomotor activating effects of dizocilpine and phencyclidine in rats. Neuropharmacology 30:629–636; 1991.
12. Miller, G.: Protein determination for large numbers of samples. Anal. Chem. 31:964; 1959.
13. Pani, L.; Kuzmin, A.; Diana, M.; De Montis, G.; Gessa, G. L.; Rossetti, Z. L.: Calcium receptor antagonists modify cocaine effects in the central nervous system differently. Eur. J. Pharmacol. 190:217–221; 1990.
14. Rafferty, M. F.; Mattson, M.; Jacobson, A. E.; Rice, K. C.: A specific acylating agent for the [^{3}H]phencyclidine receptors in rat brain. FEBS Lett. 181:318–322; 1985.
15. Reith, M. E. A.; Coffey, L. L.: [^{3}H]WIN 35,428 binding to the dopamine uptake carrier. II. Effect of membrane fractionation procedure and freezing. J. Neurosci. Methods 51:31–38; 1994.
16. Rice, K. C.; Bolger, G. T.: Interactions between phencyclidine and nifedipine at $^{45}Ca^{2+}$-uptake sites on mouse brain neurons. Biochem. Pharmacol. 39:1825–1827; 1990.
17. Rossetti, Z. L.; Pani, L.; Kuzmin, A.; Carboni, S.; Gessa, G. L.: Dihydropyridine calcium antagonists prevent cocaine-, but not amphetamine-, induced dopamine release and motor activity in rats. Acta Physiol. Hung. 75:249–250; 1990.
18. Rothman, R. B.; Mele, A.; Reid, A. A.; Akunne, H.; Greig, N.;

Thurkauf, A.; Rice, K. C.; Pert, A.: Tight binding dopamine reuptake inhibitors as cocaine antagonists. A strategy for drug development. FEBS Lett. 257:241–244; 1989.
19. Schweri, M. M.; Jacobson, A. E.; Lessor, R. A.; Rice, K. C.: Metaphit irreversibly inhibits [^{3}H]threo-(±)-methylphenidate binding to rat striatal tissue. J. Neurochem. 48:102–105; 1987.
20. Schweri, M. M.; Jacobson, A. E.; Lessor, R. A.; Rice, K. C.: Metaphit inhibits dopamine transport and binding of [^{3}H]methylphenidate, a proposed marker for the dopamine transport complex. Life Sci. 45:1689–1698; 1989.
21. Schweri, M. M.; Thurkauf, A.; Mattson, M. V.; Rice, K. C.: Fourphit: A selective probe for the methylphenidate binding site on the dopamine transporter. J. Pharmacol. Exp. Ther. 261:936–942; 1992.
22. Sershen, H.; Berger, P.; Jacobson, A. E.; Rice, K. C.; Reith, M. E. A.: Metaphit prevents locomotor activation induced by various psychostimulants and interferes with the dopaminergic system in mice. Neuropharmacology 27:23–30; 1988.
23. Simon, P.; Dupuis, R.; Constentin, J.: Thigmotaxis as an index of anxiety in mice. Influence of dopaminergic transmissions. Behav. Brain Res. 61:59–64; 1994.
24. Snell, L. D. S.; Johnson, K.; Yi, S.; Lessor, R. A.; Rice, K. C.; Jacobson, A. E.: Phencyclidine (PCP)-like inhibition of N-methyl-D-aspartate-evoked striatal acetylcholine release, [^{3}H]-TCP binding and synaptosomal dopamine uptake by Metaphit, a proposed PCP receptor acylator. Life Sci. 41:2645–2654; 1987.
25. Wise, R. A.; Bozarth, M. A.: A psychomotor stimulant theory of addiction. Psychol. Rev. 94:469–492; 1987.
26. Zimanyi, I.; Jacobson, A. E.; Rice, K. C.; Lajtha, A.; Reith, M. E. A.: Long-term blockade of the dopamine uptake complex by metaphit, an isothiocyanate derivative of phencyclidine. Synapse 3:239–245; 1989.