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GBR12909 Antagonizes the Ability of Cocaine to Elevate Extracellular Levels of Dopamine

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ROTHMAN, R. B., A. MELE, A. A. REID, H. C. AKUNNE, N. GREIG, A. THURKAUF, B. R. DE COSTA, K. C. RICE AND A. PERT. *GBR12909 antagonizes the ability of cocaine to elevate extracellular levels of dopamine.* PHARMACOL BIOCHEM BEHAV **40**(2) 387–397, 1991.—Rats were administered various IP doses of the high-affinity dopamine (DA) reuptake inhibitor 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]piperazine (GBR12909). The caudate nuclei were removed 60 min after drug administration and stored at −70°C. Striatal membranes were prepared later. The results demonstrated that GBR12909 produced a dose-dependent decrease in the binding of [^{3}H]cocaine or [^{3}H]GBR12935 to the DA transporter (ED_{50} about 10 mg/kg). Saturation binding studies with [^{3}H]GBR12935 showed that this was due to both an increase in the K_d, due to residual drug, and to a decrease in the B_{max}. At a dose of 25 mg/kg IP, GBR12909 produced a 50% decrease in the B_{max}, and a 3.4-fold increase in the K_d. In the in vivo microdialysis studies, GBR12909 (25 mg/kg IP) produced a modest, long-lasting and stable elevation of extracellular DA. Administration of cocaine through the microdialysis probe to rats pretreated with either saline or GBR12909 (25 mg/kg IP) produced a dose-dependent increase in extracellular DA in both groups. GBR12909 inhibited cocaine-induced increases in extracellular DA by about 50% at all doses. These data collectively indicate that at a dose sufficient to decrease by 50% the B_{max} of [^{3}H]GBR12935 binding sites, GBR12909 antagonizes the ability of cocaine to elevate extracellular DA by 50%. Further studies will be needed to evaluate a possible role for GBR12909 in the medical treatment of cocaine addiction.

Cocaine Dopamine Microdialysis Dopamine transport

COCAINE is a locomotor stimulant and a major drug of abuse in the United States. In addition to its action as a local anesthetic, cocaine inhibits the reuptake of norepinephrine, dopamine and serotonin, presumably by binding to a component of the macromolecular transporter complex which translocates biogenic amines from the synaptic space into the cytosol of the nerve terminal (14). Although the sympathomimetic effects of cocaine result from its inhibition of NE reuptake, its inhibition of DA reuptake is thought to be the major mechanism responsible for its euphoric and addictive effects (39).

There is, moreover, considerable evidence implicating mesolimbic DA as a crucial neurochemical mediator of rewarding behaviors such as eating and sexual behavior (48). Using the technique of in vivo microdialysis, several studies have demonstrated that the levels of extracellular DA (ECDA) increase in the nucleus accumbens of animals engaged in rewarding behaviors such as eating (19), or self-administering cocaine (22). Indeed, the elegant studies of Di Chiara et al. (13) suggest that a crucial property of drugs of abuse is their ability to elevate mesolimbic ECDA levels.

Developing medications to treat cocaine addiction has been identified as a national priority. One strategy for developing a cocaine antagonist is to identify a drug which will bind to the DA transporter, but not inhibit DA reuptake. Such a drug would, therefore, be expected to block the ability of cocaine to elevate ECDA. The development of a competitive antagonist would undoubtedly provide an important research tool. However, such a medication might have limited therapeutic uses, for a patient could overcome its inhibition by self-administering more cocaine, thereby increasing the probability of increased toxic side effects, which would not be blocked by the competitive antagonist. An alternative approach is to develop an agent which binds with high affinity to, and dissociates slowly from, the DA transporter. If the dissociation rate were slow enough, the agent would behave as a noncompetitive inhibitor, creating insurmountable inhibition of effects of cocaine mediated via elevation of ECDA. A sustained increase in ECDA produced by such an agent might serve to provide the cocaine addict with some relief from cocaine-craving, which is though to be related to a relative deficiency of DA (12).

These, and additional considerations (see below), led us to investigate the high-affinity inhibitor of DA reuptake, 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]piperazine (GBR12909) (2,45), as a possible prototypical noncompetitive

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inhibitor. Published data had shown that: 1) GBR12909 is about 700-fold more potent than cocaine in inhibiting DA reuptake in vitro ($IC_{50} = 1$ nM) (2); 2) GBR12909 is a selective inhibitor of DA reuptake (2); and 3) that its behavioral profile is somewhat different from that of other locomotor stimulants (32). Since high affinity ligands often have slow dissociation rates, and since administration of GBR12909 to rats was reported to produce only a modest increase in the levels of extracellular DA (ECDA) (41,47) it seemed worthwhile to investigate GBR12909 as a possible noncompetitive antagonist.

These considerations led us to test the hypothesis that administration of GBR12909 to rats might attenuate the ability of cocaine to elevate ECDA levels (41). The present report expands on the data published in that preliminary report, and provides additional evidence that administration of GBR12909 attenuates the ability of cocaine to increase ECDA levels in rat caudate.

METHOD

Ex Vivo Experiments

Male Sprague-Dawley rats (200 to 250 g) received IP injections of GBR12909 or vehicle (saline:DMSO 1:1, 1 ml/kg), and were sacrificed at various time points afterward. The caudate nuclei were dissected out, and stored at −70°C. On the day of the assay, the caudates of one rat were homogenized with a polytron in 5 ml ice-cold 55.2 mM sodium phosphate buffer, pH 7.4 (buffer A), and centrifuged at 39,000 × g for 10 min. The pellets were washed twice by resuspension in the same volume of ice-cold buffer A and recentrifugation. Pellets were resuspended with ice-cold 25 mM sodium phosphate buffer (buffer B) for the [^{3}H]cocaine binding assay, and in ice-cold buffer A for the [^{3}H]GBR12935 binding assay. Rats which received injections of GBR12909 demonstrated increased locomotor activity, which we did not quantitate. Rats receiving vehicle did not.

Ligand Binding Assays

To label the DA transporter, either [^{3}H]1-[2-(diphenyl-methoxy)ethyl]-4-(3-phenylpropyl)piperazine ([^{3}H]GBR12935) or [^{3}H]cocaine were used. Briefly, 100 μl of [^{3}H]ligand [in a protease inhibitor cocktail, containing 55.2 mM sodium phosphate (buffer), pH 7.4 with 25 μg/ml leupeptin, 25 μg/ml chymostatin, 0.1 mM EDTA and 0.1 mM ethyleneglycol-bis-(beta-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA)], 100 μl of either drug or buffer, and 800 μl of striatal membranes in ice-cold buffer were added to 12 × 75 mm polystyrene test tubes (total volume 1.0 ml). For GBR12909 or GBR12935 inhibition curves, serial dilutions were made up in buffer containing 1 mg/ml bovine serum albumin. [^{3}H]GBR12935 assays used buffer A, and [^{3}H]cocaine assays used buffer B. The incubations proceeded for 2–4 h at 0°C ([^{3}H]GBR12935 binding) or for 30 to 60 min at 0°C ([^{3}H]cocaine binding), at which point control experiments indicated that equilibrium was achieved. Nonspecific binding was determined using 10 μM GBR12909 for the [^{3}H]GBR12935 assay, and 10 μM cocaine for the [^{3}H]cocaine assay. Protein concentrations were determined as described by Lowry et al. (28), and consisted of 20 to 40 μg of membrane protein per assay tube ([^{3}H]GBR12935 assay) or 1 mg of membrane protein per assay tube ([^{3}H]cocaine assay). Triplicate samples were filtered on Whatman GF/B filters presoaked in buffer containing 2% polyethylenimine ([^{3}H]GBR12935 assay) or 1% polyethylenimine ([^{3}H]cocaine assay), and washed with two 5-ml aliquots of ice-cold buffer A ([^{3}H]GBR12935 assay) or ice-cold buffer B containing 0.4 M NaCl ([^{3}H]cocaine assay). The [^{3}H]GBR12935 assays were filtered using a Brandell MR24 cell harvester, whereas the [^{3}H]cocaine assays were filtered one sample at a time using a single manifold. Filters were placed in scintillation vials prefilled with 10 ml of Beckman Ready Safe scintillation fluid, and the tritium retained on the filters counted the next day.

Supernatant Experiments

In other experiments, supernatants collected during the preparation of striatal membranes were saved. The supernatant resulting from the initial centrifugation of the homogenate was called the S1. The supernatants resulting from the first, second and third washes were termed the S2, S3 and S4, respectively. Inhibitory activity present in the supernatant was determined by assaying the ability of the supernatant to inhibit [^{3}H]GBR12935 binding to caudate membranes prepared from untreated rats. For these experiments, the assay tubes received the following additions: 100 μl of [^{3}H]GBR12935, 100 μl of buffer or drug, 750 μl of supernatant, and 100 μl of control membranes. The concentration of inhibitory activity was quantified by comparison with a GBR12935 inhibition curve which was made up with supernatant prepared from control membranes.

To determine the approximate concentration of GBR12909 in the brain, rats were administered GBR12909 (25 mg/kg, IP) or vehicle and sacrificed 1 h later. The whole brain (including cerebellum) was homogenized in 10 ml ice-cold 55.2 mM sodium phosphate (buffer), pH 7.4, and centrifuged at 11,953 × g for 10 min. The supernatant (S1) was frozen, and 50-μl aliquots later assayed for inhibition of [^{3}H]GBR12935 binding. Comparison was made to a GBR12909 displacement curve which was made up using control supernatant.

Experimental Design and Data Analysis

To determine the K_d and B_{max} of [^{3}H]GBR12935 binding site(s), the method of binding surface analysis was used (40). [^{3}H]GBR12935 binding surfaces were generated by displacing two concentrations of [^{3}H]GBR12935 by concentrations of nonradioactive GBR12935 (0.25 to 64 nM). In other experiments, binding surfaces of other agents were generated by displacing two concentrations of [^{3}H]GBR12935 by various concentrations of the agent. Data were fit for the best-fit parameter estimates to one, two or three site binding models using MLAB (27), which uses a weighted nonlinear least squares curve fitting algorithm. In all cases, consideration of a two or three binding site model did not reduce the sum-of-squares to a statistically significant degree, as determined with the F-test (31). Statistical significance among the best-fit parameter estimates of control and experimental groups was assessed (31) by fitting the data of all groups (control and experimental simultaneously) with the constraint that a parameter of the control group is equal to the corresponding parameter of the experimental group. This reduces the number of fitted parameters by 1, and increases the degrees of freedom by one. The increase in the SS produced by the constraint is then used to calculate an F value as described above. If the corresponding p value is less than 0.05, then the parameters are assumed to be different.

In Vivo Microdialysis

Male Sprague-Dawley rats (300–350 g, Taconic Farms, MD) were anesthetized with chloral hydrate 400 mg/kg and placed in a stereotaxic frame. Anesthesia was maintained at a level throughout each experiment whereby corneal reflexes were abolished. A 3-mm microdialysis probe (Carnagie Medicine) was

lowered into the striatum at stereotaxic coordinates of AP +9.9, ML +3.2, DV +3.0 (34). Artificial CSF (aCSF) containing NaCl (8.6 g), KCl (0.3 g), $CaCl_2 \cdot 2H_2O$ (0.335 g) and ascorbic acid (0.004 g) in distilled water (1 l) was pumped through the probe at a flow rate of 1.0 μl/min using a microinfusion pump (CMA/100). After the probe was inserted, 20-μl samples were collected every 20 min into polyethylene tubes containing 10 μl perchloric acid (1 M). Immediately after collection samples were injected into a high pressure liquid chromatography system and analyzed using electrochemical detection. After two hours, 3 baseline samples were collected and then the pharmacological treatment was started.

Treatment consisted of either GBR12909 (25 mg/kg) or saline injected IP. One h afterwards the same rats were infused through the probe with (−)-cocaine (0.1, 1.0 or 10.0 mM) dissolved in aCSF. After 20 min, perfusion of the probe with normal aCSF was resumed and samples were collected for an additional hour.

The HPLC system used in these studies included a BAS LC-4B electrochemical detector with a glassy carbon working electrode set at 0.730 V. A Brownlee 10 cm reversed phase C18 column, and a mobile phase comprised of sodium acetate (2.0 g), citric acid (3.0 g), sodium octylsulphate (350 mg), EDTA (88 mg) and methanol (70 ml) in distilled water (1 l) were used to isolate the DA in the samples.

Statistical analysis of the data used analysis of variance with a post hoc Scheffe test.

Chemicals

[^{3}H]Cocaine and [^{3}H]GBR12935 were purchased from New England Nuclear. GBR12909 and GBR12935 were synthesized in the Laboratory of Medicinal Chemistry as described elsewhere (45). (−)-Cocaine was obtained from NIDA.

RESULTS

Ex Vivo Experiments

Initial studies demonstrated that administration of GBR12909 at a dose of 40 mg/kg to rats produced a 77.3% and 55.0% decrease in [^{3}H]GBR12935 binding (2 nM) 1 h and 4 h after drug administration (data not shown). In subsequent experiments rats were sacrificed 1 h after drug administration. Administration of various doses of GBR12909 produced an almost identical dose-dependent decrease in both [^{3}H]cocaine and [^{3}H]GBR12935 binding (Fig. 1). The ED_{50} values were about 10 mg/kg for both [^{3}H]ligands.

To determine the effect of GBR12909 on the K_d and B_{max} of the DA transporter, [^{3}H]GBR12935 binding surfaces were generated by displacing two concentrations of [^{3}H]GBR12935 (0.5 and 2.5 nM) by nonradioactive GBR12935. The binding surfaces were best fit by a one-site binding model (data not shown). These data demonstrated that administration of GBR12909 produced both an increase in the K_d (Fig. 2A) and a decrease in the B_{max} (Fig. 2B) of [^{3}H]GBR12935 binding sites. The maximal decrease in the B_{max} was about 50%. The best-fit binding parameters are reported in Table 1.

To determine the cause of the increased K_d value, caudate membranes from rats which received 25 mg/kg GBR were washed several times by resuspension and recentrifugation. The supernatants were retained, and later assayed for their ability to inhibit [^{3}H]GBR12935 binding to control caudate membranes. As reported in Table 2, an equal amount of inhibitory activity was present in all the supernatants, demonstrating that the increase in the K_d was most likely due to GBR dissolved in the membranes which partitions into the aqueous phase every time a membrane pellet is resuspended.

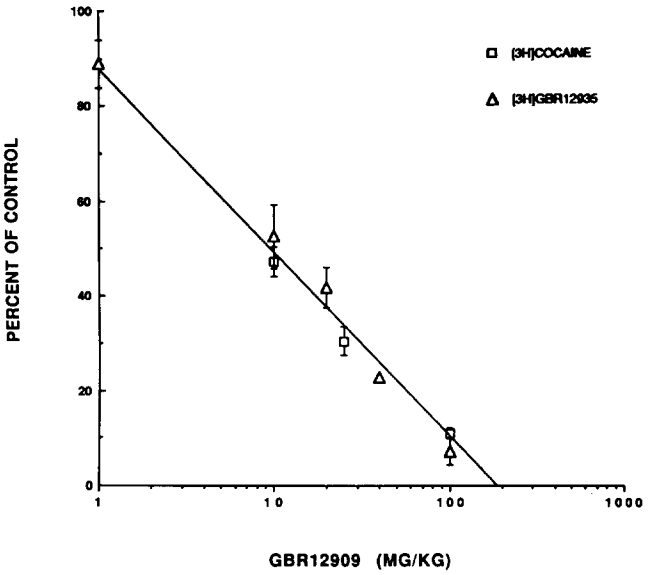

FIG. 1. The indicated doses of GBR were administered to rats, which were sacrificed 1 h after the injection. Striatal membranes were prepared and [^{3}H]cocaine and [^{3}H]GBR12935 binding determined as described in the Method section. Each point is the mean ± SD (N = 4). [*p<0.01 when compared to control (post hoc Scheffe F-test).] All data points were significantly different from control (except 1 mg/kg).

Similar experiments were performed with GBR12935. As reported in Table 3, IP administration of GBR12935 resulted in a dose-dependent increase in the K_d of [^{3}H]GBR12935 binding sites, without any significant change in the B_{max} values, as well as a dose-dependent increase in the inhibitory activity of the supernatant. Administration of benztropine (50 mg/kg) had no significant effect on [^{3}H]GBR12935 binding (data not shown).

To rule out the possibility that the GBR12909-induced decrease in the [^{3}H]GBR12935 B_{max} was not due to nonspecific effects, rats were pretreated with mazindol (30 mg/kg) prior to administration of GBR12909. The results (Table 4) demonstrated that mazindol by itself did not affect [^{3}H]GBR12935 binding, but did partially reverse the GBR12909-induced decrease in the B_{max}, thereby demonstrating that occupation of the transporter by GBR12909 is required to produce the decrease in the B_{max}.

The supernatant experiments described above measured GBR12909/GBR12935-like inhibitory activity present in supernatants prepared from striatal membranes. That is, the caudates had already been homogenized, and the first supernatant discarded. To get a better idea of the brain concentration of GBR12909 produced by IP administration of 25 mg/kg of this agent, rats were administered this dose and sacrificed 1 h later. The brains from injected animals were homogenized, centrifuged, and the inhibitory activity present in the first supernatant measured. As reported in Table 5, a substantial amount of inhibitory activity was detected in 50 μl of the initial supernatant. The total amount of GBR12909 present in the initial supernatant was calculated to be 20 nmol. Assuming an even distribution of GBR12909 throughout the brain, and that a rat brain has about 1.4 ml of water, the approximate concentration of GBR12909 in the brain can be calculated to be about 14 μM.

[^{3}H]GBR12935 has been reported to label a "piperazine acceptor site," for which cis-flupentixol has relatively high affinity (IC_{50} = 240 nM) (1). To see if [^{3}H]GBR12935 labeled the

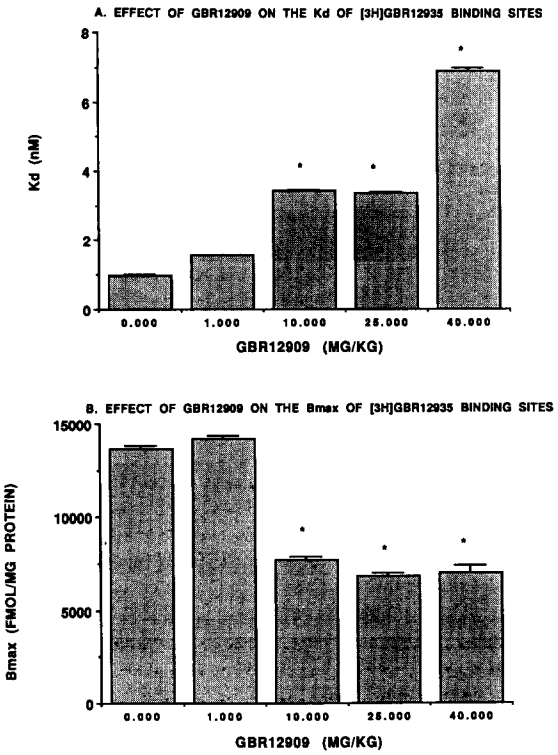

FIG. 2. (A and B) The indicated doses of GBR12909 were administered to rats, which were sacrificed 1 h after the injection. Striatal membranes were prepared and [^{3}H]GBR12935 binding surfaces were generated by displacing two concentrations of [^{3}H]GBR12935 (0.5 and 2.5 nM) each by eight concentrations of GBR12935. The data were fit to a one-site binding model for the best-fit parameter estimates (±SD). *$p<0.05$ when compared to control (F-test).

piperazine acceptor site, we studied the interaction of benztropine and cis-flupentixol with [^{3}H]GBR12935 binding sites. Labeling of the piperazine acceptor site should be detected as the presence of a second site with high affinity for cis-flupentixol and low affinity for benztropine. GBR12935, benztropine and cis-flupentixol binding surfaces (Fig. 3) were fit to one- and two-site binding models, with no improvement in the goodness-of-fit produced by the two-site model. The best-fit parameter estimates, reported in Table 6, were GBR12935 ($K_d = 1.51$ nM), benztropine ($K_i = 219$ nM) and cis-flupentixol ($K_i = 1226$ nM), consistent with the hypothesis that [^{3}H]GBR12935 does not label the piperazine acceptor site using our experimental conditions.

TABLE 2

EFFECT ON WASHING MEMBRANES BY CENTRIFUGATION ON THE INHIBITORY ACTIVITY OF THE SUPERNATANTS

Supernatant	Percent Inhibition of [^{3}H]GBR12935 Binding
S2	42.4
S3	49.2
S4	52.8

Rats were administered vehicle or GBR12909 (25 mg/kg) and, as described in the Method section, supernatants (700 µl) were collected during the preparation of striatal membranes. They were then assayed for inhibition of [^{3}H]GBR12935 binding (2 nM). Supernatants from the vehicle-injected rats served as the control. The SD values were less than 10 percent of the value.

In Vivo Microdialysis Studies

ECDA levels were quantitated as a percent of the predrug baseline. Administration of GBR12909 (25 mg/kg) to rats produced a modest increase (about 200 percent of control) in ECDA levels (Fig. 4), which stabilized 60 min after drug administration, and remained constant at that level for at least 3 additional h. Analysis of variance demonstrated a statistically significant difference between the saline and GBR12909 groups, although only two time points were different to a statistically significant degree.

Administration of cocaine through the microdialysis probe to rats injected with either saline or GBR12909 (25 mg/kg) demonstrated a statistically significant decrease in the ability of three doses of cocaine to elevate ECDA above the predrug baseline

TABLE 1

EFFECT OF IN VIVO ADMINISTRATION OF GBR12909 ON [^{3}H]GBR12935 BINDING SITES IN VITRO

Dose (mg/kg IP)	B_{max} (±SD) (fmol/mg protein)	K_d (±SD) (nM)	r^2	(GBR12909) (nM±SD) Measured	Calculated
0	13666 ± 573	0.98 ± 0.07	.97	0	0
1	14195 ± 590	1.57 ± 0.08*	.98	0	0.59
10	7685 ± 725*	3.43 ± 0.15*	.98	3.6 ± 0.2	2.45
25	6780 ± 782	3.36 ± 0.16	.98	ND	2.38
40	6962 ± 1819*	6.93 ± 0.43*	.96	14.0 ± 0.9	5.95
100	ND	ND	ND	50.5 ± 12.0	ND

As described in the Method section, rats received IP injections of GBR12909, and were sacrificed 1 h later. Striatal membranes were prepared, and the S2 supernatant was retained. Saturation binding experiments with [^{3}H]GBR12935 permitted the determination of the K_d and B_{max} of the DA transporter. The best-fit parameter estimates are reported above, along with the correlation coefficient, r^2. The apparent concentration of GBR12909 in the S2 was measured as described in the Method section. This value is compared to that calculated on the basis of the increased K_d value produced by administration of GBR12909. "ND" means "not determined." $p<0.05$ when compared to control (F-test).

TABLE 3

EFFECT OF IN VITRO ADMINISTRATION OF GBR12935 ON [^{3}H]GBR12935 BINDING SITES IN VITRO

Dose (mg/kg IP)	B_{max} (±SD) (fmol/mg protein)	K_d (±SD) (nM)	r^2	(GBR12935) (nM) Measured	Calculated
0	10,500 ± 374	1.16 ± 0.06	.98	0	0
0.2	11,885 ± 296*	1.28 ± 0.04	.99	0	0.12
1.0	12,717 ± 488*	1.79 ± 0.08*	.98	0	0.63
10	11,081 ± 397	2.46 ± 0.08*	.98	1.8 ± 0.1	1.3
20	10,538 ± 506	2.77 ± 0.10*	.98	3.5 ± 0.3	1.6
40	12,490 ± 956	4.08 ± 0.17*	.98	4.3 ± 0.9	2.92
80	9430 ± 173	8.99 ± 0.41*	.98	15.9 ± 1.8	7.83

As described in the Method section, rats received IP injections of GBR12935, and were sacrificed 1 h later. Striatal membranes were prepared, and the S2 supernatant was retained. Saturation binding experiments with [^{3}H]GBR12935 permitted the determination of the K_d and B_{max} of the DA transporter. The best-fit parameter estimates are reported above, along with the correlation coefficient, r^2. The apparent concentration of GBR12935 in the S2 was measured as described in the Method section. This value is compared to that calculated on the basis of the increased K_d value produced by administration of GBR12935. *$p<0.05$ when compared to control (F-test).

(Fig. 5). The increase in ECDA levels produced by cocaine was calculated as the ECDA level in the presence of cocaine minus the ECDA level in the absence of cocaine. As reported in Fig. 6, administration of GBR12909 substantially inhibited the ability of cocaine to increase ECDA levels at all three cocaine concentrations.

DISCUSSION

Evidence That GBR12909 Binds Tightly to the DA Transporter

Administration of GBR12909 to rats produced a dose-dependent decrease in both [^{3}H]GBR12935 and [^{3}H]cocaine binding (Fig. 1). The saturation binding studies demonstrated that this was due to both a decrease in the B_{max}, and an increase in the K_d of [^{3}H]GBR12935 binding sites (Fig. 2). That the increase in the K_d value is due to residual drug is clearly indicated by the direct measurement of inhibitory activity in the supernatants prepared from the striatal membranes of GBR12909-treated rats. Moreover, the inhibitory activity was high enough to account for the increased K_d value. Interestingly, washing the membranes by repeated resuspension and centrifugation did not reduce the amount of inhibitory activity. This suggests that GBR12909 dissolves in the membranes, and that each time the membrane pellet is resuspended in fresh buffer, some of it partitions into the aqueous buffer, resulting in an inhibition of [^{3}H]GBR12935 binding. In addition, data reported here that administration of GBR12909 to rats produces identical decreases in both [^{3}H]GBR12935 and [^{3}H]cocaine binding is consistent with the hypothesis that [^{3}H]GBR12935 and [^{3}H]cocaine label a common binding site.

The observation that administration of GBR12909 decreases the B_{max} of [^{3}H]GBR12935 binding sites is most simply explained by an apparently persistent occupation of that binding site by GBR12909. A more convincing demonstration would be to take membranes prepared from GBR12909-treated rats through a protocol known to increase the dissociation rate of GBR12909, and show that this procedure also increased the number of [^{3}H]GBR12935 binding sites. Unfortunately, such a protocol (lowering the pH to 5.0) also causes an irreversible decrease in binding (unpublished data). An alternative approach would be to co-administer GBR12909 with another agent which does not produce a decrease in the B_{max}. If the decrease in the B_{max} is due to an apparent persistent occupation of the DA transporter by GBR12909, then the second agent should partially block the GBR12909-induced decrease in the B_{max}. As reported

TABLE 4

EFFECT OF MAZINDOL ON GBR12909-INDUCED DECREASES IN THE B_{max} OF THE DA TRANSPORTER

Condition	B_{max} (percent of control)	K_d (percent of control)
Control	100	100
GBR12909	35 ± 2*	93 ± 7
GBR12909+ Mazindol	61 ± 4*†	126 ± 6*

Rats were administered saline or mazindol (30 mg/kg IP) at time −15 min. At time 0, rats received IP injections of either GBR12909 (10 mg/kg) or vehicle. The rats were sacrificed at time 30 min. The caudates were dissected and kept frozen at −70 °C until assayed as described in the Method section. Mazindol by itself did not affect [^{3}H]GBR12935 binding. *$p<0.05$ when compared to control. †$p<0.05$ when compared to the GBR12909 condition (F-test).

TABLE 5

BRAIN CONCENTRATION OF GBR12909 FOLLOWING ADMINISTRATION OF 25 mg/kg

Supernatant	Percent Inhibition of [^{3}H]GBR12935 Binding (±SD)	nmols of GBR12909 in the Brain
S1 (50 µl)	39.5 ± 8.5	20 ± 4 nmol (approx. 14 µM)

Rats were administered vehicle or GBR12909 (25 mg/kg), as described in the Method section. Following homogenization in 10 ml ice-cold 55.2 mM sodium phosphate (buffer), pH 7.4, and centrifugation, the supernatants were retained and 50 µl aliquots subsequently assayed for inhibition of [^{3}H]GBR12935 binding (2 nM). Supernatants from the vehicle-injected rats served as the control. Each value is the mean ± SD (n = 4 rats).

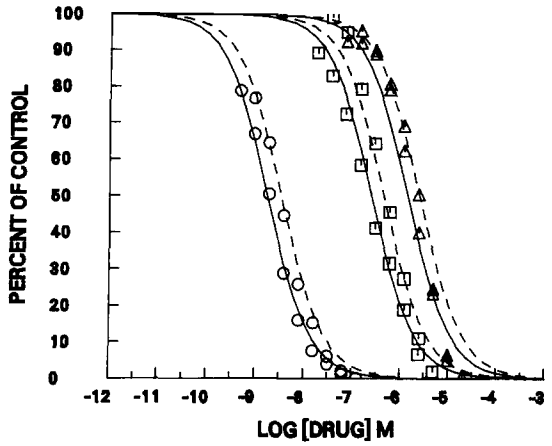

FIG. 3. Two concentrations of [^{3}H]GBR12935 (0.5 and 2.5 nM) were each displaced by 8 concentrations of GBR12935 (open circles), benztropine (open squares) and cis-flupentixol (open triangles). The data were combined and fit to one-site (SS = 0.176) and two-site (SS = 0.176) binding models. The best-fit parameter estimates of the one-site binding model generated the lines in the figure.

in Table 4, mazindol partially blocked the GBR12909-induced decrease in the B_{max}.

Although 1 mg/kg of GBR12909 increased the K_d of [^{3}H]GBR12935 binding sites, it had no significant effect on the B_{max}. The 10, 25 and 40 mg/kg doses of GBR12909 all decreased the B_{max} by about 50%. One interpretation of this observation is that the striatum possesses two classes of DA transporters, and that GBR12909 dissociates very slowly from one class, and much faster from the second class, a notion which is consistent with the findings of Graybiel et al. (17) who reported that the DA transporter was differentially distributed between the striatal matrix and the striasomes.

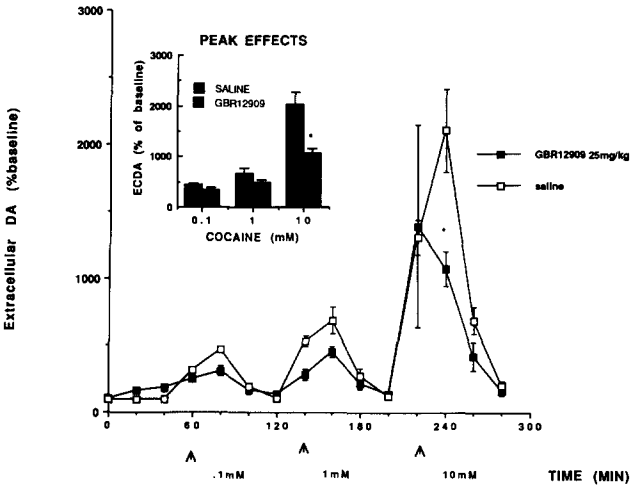

FIG. 5. Rats were administered either saline or GBR12909 (25 mg/kg). Cocaine was administered through the microdialysis probe, and the ECDA levels calculated as a percent of the predrug baseline. Analysis of variance demonstrated a significant difference between treatment groups. *$p<0.05$ when compared to control (post hoc Scheffe F-test).

Unlike GBR12909, administration of GBR12935 to rats did not decrease the B_{max} of [^{3}H]GBR12935 binding sites. Since both agents have about the same K_d for the [^{3}H]GBR12935 binding site (1), these data suggest that the pharmacophore for tight binding might be different from the pharmacophore for high-affinity binding. It will be interesting to examine, in future studies, the effect of GBR12935 on cocaine-induced increases in

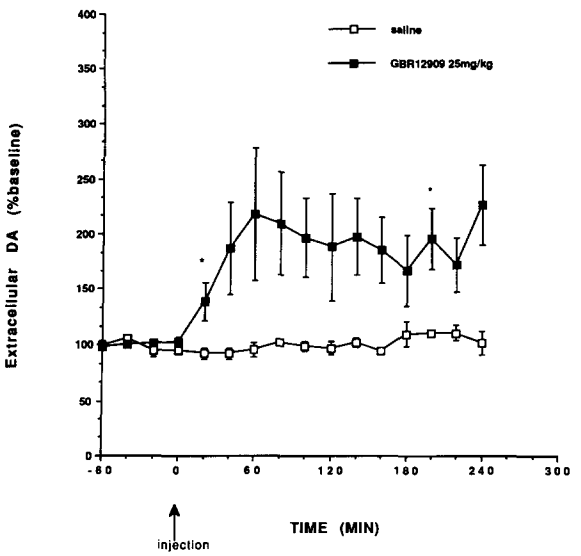

FIG. 4. In vivo microdialysis studies were conducted as described in the Method section. After a 60-min equilibration period, drugs were administered at time 0, and ECDA levels (calculated as a percent of the predrug baseline) measured in 20-min samples. Each point is the mean ± SD of 5 or 6 experiments. Analysis of variance demonstrated a significant difference ($p<0.05$) between treatment groups. *$p<0.05$ when compared to control (post hoc Scheffe F-test).

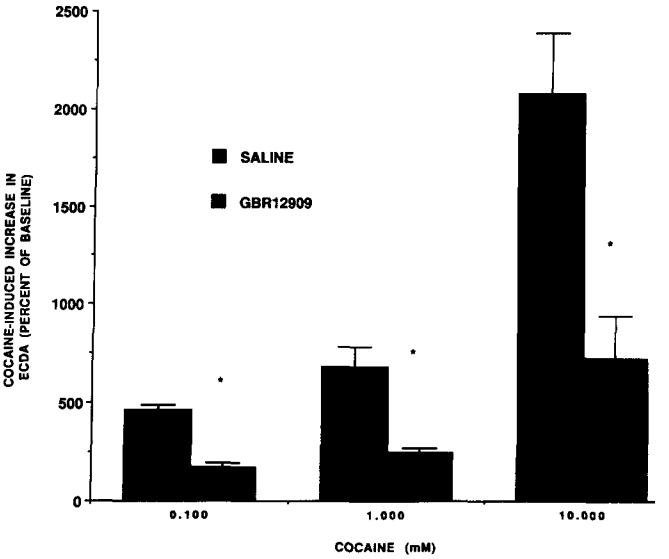

FIG. 6. The increase in ECDA levels produced by cocaine in Fig. 5 (the peak effect) was calculated according to the following equation: [ECDA levels produced by cocaine] = [ECDA level in the presence of cocaine] − [ECDA level in the absence of cocaine]. The ECDA level in the absence of cocaine was defined as the ECDA level in the 20-min sample preceding the administration of cocaine. Thus the increase was calculated relative to the preexisting baseline. *$p<0.05$ when compared to control (post hoc Scheffe F-test).

TABLE 6

BEST-FIT PARAMETER ESTIMATES OF THE [^{3}H]GBR12935 BINDING SURFACES

Parameter	Value (± SD)
B_{max} (fmol/mg) (protein)	10714 ± 760
GBR12935 (K_d, nM)	1.51 ± 0.17
Benztropine (K_i, nM) (K_i, nM)	219 ± 19
cis-Flupentixol (K_i, nM) (K_i, nM)	1226 ± 109

GBR12935, benztropine and cis-flupentixol binding surfaces were generated as described in the Method section, by displacing two concentrations of [^{3}H]GBR12935 (0.5 and 2.5 nM) each by eight concentrations of inhibitor. The combined data of two experiments were fit to a one-site binding model (SS = 0.149), and to a two-site binding model (SS = 0.149). The best-fit parameter estimates of the one-site binding model are reported above.

ECDA. The lack of effect of benztropine (50 mg/kg) on [^{3}H]GBR12935 binding may seem somewhat surprising, in that lower doses (25 mg/kg) substantially increase ECDA levels (11). The likely explanation is that unlike GBR12935, there is not enough residual drug in the membranes to produce a detectable inhibition of [^{3}H]GBR12935 binding.

Recognition of the apparently low in vivo potency of GBR12909 to increase ECDA in relation to its high potency to inhibit [^{3}H]DA reuptake in vitro led Menacherry et al. (29) to hypothesize that this was due to poor penetration of GBR12909 into the brain. In support of this hypothesis, they demonstrated that administration of 100 mg/kg of GBR12909 IP resulted in a relatively low concentration of GBR12909 in a dialysate of extracellular fluid (250 nM). This is different from the results of the present study, which demonstrate that a dose of GBR12909 as low as 10 mg/kg IP results in apparently tight binding of GBR12909 to about 50% of the DA transporters, an observation which clearly demonstrates that this dose of GBR12909 readily distributes into the brain. Moreover, our data indicate that administration of 25 mg/kg of GBR12909 achieves a brain concentration of about 14 μM. In support of this finding, Scheffel et al. reported that 10 mg/kg of GBR12909 administered IV almost completely inhibited the in vivo binding of [^{3}H]WIN-35,065-2 to the DA transporter (42). Moreover, we have observed that administration of 25 mg/kg of GBR12909 IP almost completely inhibits the in vivo binding of [^{3}H]N-[1-(2-benzo(b)thiophenyl)cyclohexylpiperidine ([^{3}H]BTCP) to the DA transporter (unpublished data). The likely explanation for the discrepancy between our findings and that of Menacherry et al. (29) is that whereas they measured the GBR12909 present in the extracellular fluid of the rat brain, we measured GBR12909-like inhibitory activity in a whole brain supernatant. Given the high lipid solubility of GBR12909, and its demonstrated ability to partition into brain membranes (Table 2), it is likely that only a small fraction of the GBR12909 in the brain is detectable in a dialysate of the extracellular fluid. Moreover, it is not possible to tell to what extent redistribution of drug from the lipid compartment to the aqueous compartment during homogenization influenced our results. It is also important to point out that the method used in this study to detect GBR12909 will detect active metabolites, whereas the method used by Menacherry et al. (29) (thermospray tandem mass spectroscopy) detects GBR12909 only. Unfortunately, there are no published data of which we are aware which speaks to the existence or biological activity of GBR12909 metabolites. Given the high lipid solubility of GBR12909, and the low levels of GBR12909 found in the extracellular fluid, it is likely that the free concentration of GBR12909 is considerably less than 14 μM. Nevertheless, the data presented in this study clearly indicate that GBR12909 does readily penetrate into the brain.

Effect of GBR12909 on Extracellular DA

For the in vivo microdialysis studies, GBR12909 was administered systemically at a dose of 25 mg/kg. As described above, this dose of GBR12909 apparently persistently occupied at least 50% of striatal DA transporters, and achieved a concentration in the brain estimated to be about 14 μM, which is sufficient to completely inhibit DA reuptake in vitro. Consistent with its binding tightly to the DA transporter, GBR12909 produced a stable elevation of ECDA which lasted for the duration of the experiment (3 to 4 h). However, the magnitude of the increase (about 200%) was relatively small, compared to the rather large increases which can be produced by systemic administration of comparable doses of cocaine (11).

To assess the effect of GBR12909 on cocaine-induced increases in ECDA, three doses of cocaine (0.1, 1.0 and 10 mM) were sequentially administered to the same animal. Published data suggest that this protocol is unlikely to produce tachyphalaxis (20,22). Although these doses appear to be rather high, only a fraction of the dose actually crosses the dialysis membrane. In addition, the elevations of ECDA produced by these doses of cocaine reflect inhibition of reuptake, and not a nonspecific mechanism, since 10 mM (+)-cocaine does not increase ECDA (16).

Although one might have expected the effects of cocaine and GBR12909 to be additive, they were not. In fact, cocaine-induced ECDA levels were significantly lower in the GBR12909-treated rats than in control rats (Fig. 5). In this graph, the data are presented as a percent of the ECDA level prior to the administration of GBR12909. As noted above, administration of GBR12909 produced a stable increase in the ECDA level. Administration of cocaine through the microdialysis probe increased the ECDA above this baseline, and after 40 min, the ECDA had returned to the preexisting baseline value. Therefore, the cocaine-induced increase in the ECDA is more appropriately calculated as the ECDA level in presence of cocaine minus the ECDA level in the absence of cocaine. As reported in Fig. 6, GBR12909 inhibited cocaine-induced increases in ECDA by about 50% for all three doses of cocaine.

One hypothesis to explain these data is as follows. 1) At the dose used (25 mg/kg), GBR12909 binds to, and dissociates very slowly from about 50% of the DA transporters. 2) Although the brain concentration of GBR12909 is high, the free concentration of GBR12909 must be low, so that cocaine can bind to unoccupied transporters. 3) Cocaine cannot bind to transporters to which GBR12909 is already bound. 4) Cocaine increases ECDA by binding to DA transporters not occupied by GBR12909. 5) Therefore, there is about a 50% inhibition of the cocaine-induced increase in the ECDA levels. 6) If the reuptake of DA by transporters occupied by GBR12909 is inhibited to the same degree as when the transporters are occupied by cocaine, then the elevation of ECDA produced by cocaine and GBR12909 should be additive. The fact that the combined effect is less than additive suggests that transporters occupied by GBR12909 are less effectively inhibited than transporters occupied by cocaine.

One way of restating the sixth postulate is that when ECDA

is defined as the dependent variable, GBR12909 is a partial agonist. Although several studies indicate that cocaine and the GBR12909 analog GBR12783 increase ECDA by inhibiting DA reuptake (8, 20, 21), it is important to point out that it is not the dependent variable, since it is not being directly measured. Rigorously proving this postulate would require doing complete dose-response curves, which is beyond the scope of the present study, as pointed out below.

In a dose-response study, a number of controls have to be run to ensure that increases in ECDA are due to reuptake inhibition, and are not due to nonspecific effects. With systemic administration of drugs, full dose-response curves cannot be run because toxic side effects occur before maximal effects are observed. For example, the maximum dose of GBR12909 we have examined is 100 mg/kg. At this dose, the mortality is about 50%. Given that ECDA levels increase in dying animals (46), it would be almost impossible to distinguish between GBR12909-induced increases in ECDA, and the increase which would independently result from the process of dying.

Many of these issues can in principle be avoided by administration of drugs through the microdialysis probe. In fact, Hurd et al. (20) demonstrated that the ability of systemically administered cocaine to elevate striatal ECDA is due to actions in the striatum. However, increases in ECDA produced by mechanisms other than inhibition of reuptake must be carefully ruled out. This is usually done by testing each dose under conditions where nerve impulse coupled release of DA is inhibited. Commonly used agents are tetrodotoxin, Ca^{++} free buffer, or γ-butyrolactone. These agents will not block amphetamine-like effects, which could occur at any dose of an agent. In this regard, a selective irreversible ligand for the DA transporter would serve to block an increase in ECDA mediated by the transporter, and would, therefore, serve to control for amphetamine-like effects. Nevertheless, this approach would not control for inhibition of K^+ channels, which, by prolonging the action potential, would increase the release of DA. In addition, it would be important to control for the influence of the striato-nigral feedback loop and autoreceptors located presynaptically on the DA terminals: administration of a reuptake blocker via the microdialysis probe would increase synaptic levels of DA, which would decrease release via binding to these autoreceptors, and by also acting to decrease the firing rate of topographically mapped neurons in the substantia nigra, which would in turn act to decrease the effect of the reuptake blocker. These feedback loops might be controlled for by including DA receptor antagonists in the superfusate.

One mechanism to explain the apparent partial agonist effect of GBR12909 is that it inhibits the reuptake mechanism less effectively than does cocaine. This notion is not consistent with data demonstrating that GBR12909, like cocaine, is a competitive inhibitor of [^{3}H]DA reuptake in vitro (2). It is unlikely that cocaine increases ECDA via amphetamine-like effects, since its effects are blocked by the use of Ca^{++}-free medium or γ-butyrolactone (7,20). Alternatively, cocaine may have another action which potentiates its effect as a DA reuptake blocker. For example, whereas cocaine inhibits 5-HT reuptake more potently than it inhibits DA reuptake, GB12909 is about 170-fold more potent as a DA reuptake inhibitor (2). Given evidence for a role for 5-HT_3 receptors in increasing mesolimbic DA (23,25), the ability of cocaine to increase synaptic levels of 5-HT might conceivably potentiate its actions as a DA reuptake blocker. Data that administration of fluoxetine, a selective inhibitor of 5-HT reuptake, did not alter cocaine self-administration by rats argues against this hypothesis (37), although the data of Carroll argue for it (9).

Speculations on the Use of GBR12909 as a Cocaine 'Antagonist' in Humans

The observations reported in the present study that GBR12909 attenuates the ability of cocaine to elevate ECDA suggest that GBR12909 might be useful in humans as a treatment for cocaine abuse. However, there is also reason to believe that GBR12909 will act like cocaine in humans, and perhaps, be more potent than cocaine. This discussion will first address this latter possibility.

To begin with, an assumption of this and other studies which measure drug effects on ECDA, is that ECDA is a measurement relevant to the mechanism of action of the drug being studied. The level of ECDA does not directly reflect the rate of DA release per se, but DA overflow, which is the amount remaining after other mechanisms such as reuptake, diffusion, and metabolism have acted to decrease levels of ECDA. In fact, many of the behavioral effects of psychomotor stimulants occur at doses which cause minimal elevations in ECDA. Similarly, electrical stimulation of the medial forebrain bundle or the substantia nigra produces effects generally thought to be mediated by DA, but with only minimal elevations of ECDA (A. Pert, personal communication). Viewed collectively with the data presented here that 10 mg/kg of GBR12909 apparently occupies at least 50% of the DA transporters while causing minimal changes in ECDA, it seems clear that the effects on ECDA observed in this study may not be relevant to the behavioral effects of psychomotor stimulants observed in animals. In other words, even assuming that GBR12909 acts as a partial agonist, it may increase synaptic DA high enough to produce the typical effects of a psychomotor stimulant. Indeed, GBR12909 is self-administered by rhesus monkeys (Woods, J. W., personal communication) and stimulates locomotor activity as effectively as does cocaine (C. Hubner, personal communication).

On the other hand, it is also possible to argue that GBR12909, or a drug acting via a similar mechanism, might be useful for treating cocaine abuse in humans. Behavioral paradigms in which animals are trained to self-administer drugs via the intravenous route are commonly accepted to predict the abuse liability of a drug in humans. It is generally believed that the only exceptions to this "rule" are the hallucinogens, which are abused by humans, but are not self-administered by animals. Clearly, this belief is an oversimplification of a complex behavioral assay. If this belief were true, i.e., completely validated, then the self-administration paradigm would be one of the few diagnostic tests in medicine without any false positives, i.e., drugs which are self-administered by animals but which are not abused by humans. However, Iwamoto and Martin pointed out that there are numbers of false positives (24). They also point out that the term "abuse" is a loosely defined concept, which not only requires that the drug be intrinsically reinforcing, but that the potential "abusers" of the drug have access to it, and that prevailing attitudes and existing drug subcultures encourage the abuse of a given drug. Therefore, for the purposes of this discussion, and in the context of the psychomotor stimulants, which produce in humans the subjective experience described as euphoria, we define a false positive drug as one which is self-administered by animals, but which does not produce euphoria in humans.

As discussed by Iwamoto and Martin (24), there are many examples of drugs which are self-administered by animals, but not abused by humans. Among the opioids, ketocyclazocine, ethylketocyclazocine and metazocine support self-administration behavior in animals. Among dopaminergic agonists, apomorphine, piribidel and bromocriptine support self-administration in animals. The $alpha_2$ andrenergic agonist, clonidine, which is

widely used as an antihypertensive medication, is self-administered by rhesus monkeys. There are, to our knowledge, no reports of clonidine-induced euphoria. Short-acting local anesthetics such as procaine, chloroprocaine, dimethylprocaine, and dimethocaine are also self-administered by animals. In addition, several local anesthetics such as procaine and lidocaine are self-administered by animals, but are not known to produce euphoria in humans, although large IV doses of procaine were identified as cocaine by three human subjects.

More relevant to the possible use of GBR12909 as a treatment for cocaine abuse is the lack of reports that the DA reuptake blockers bupropion, nomifensine, and mazindol produce euphoria in humans. Bupropion is marketed as an antidepressant, and is about as potent as cocaine in inhibiting DA reuptake in vitro (1). Mazindol is marketed as an anorectic, and is 23 times more potent than cocaine in inhibiting DA reuptake (1). Nomifensine was marketed as an antidepressant prior to its removal from the market due to serious allergic side effects, is 5 times more potent than cocaine as a DA reuptake blocker (1), and was used to diagnose hyperprolactsemia (36).

Data supporting the assertion that these agents, which are not controlled substances (with the exception of mazindol), do not produce euphoria in humans following oral administration are the facts that 1) the prescribing literature for these medications makes no mention of euphoria as a possible side effect; 2) mazindol has been documented to produce in humans subjectively unpleasant effects (10); 3) where the subjective effects of bupropion and nomifensine were specifically examined, there was no mention of drug-induced euphoria (18, 30, 33, 35, 44, 49); 4) the common sense notion that if cocaine were as widely prescribed as these drugs have been, then the euphoric effects of cocaine would have been obvious. Regarding the third point, the studies of Peck and Hamilton (35) showed that oral administration of 200 mg of bupropion or 100 mg of nomifensine did not produce amphetamine-like CNS stimulant activity in normal volunteers. Moreover, the study of Miller and Griffith (30) demonstrated that bupropion at oral doses up to 400 mg did not produce amphetamine-like subjective effects in experienced abusers of amphetamine.

There are several possible explanations for why these DA reuptake blockers do not produce euphoria in humans, yet are self-administered by animals. The most obvious explanation is the route of administration. Self-administration studies in animals use the intravenous route, whereas nomifensine, bupropion and mazindol are taken orally by humans. Intravenous administration of cocaine produces very rapid delivery of drug to the brain, which is followed by rapid clearing. This type of pharmacokinetic pattern has been suggested to be important for the euphoric and addictive effects of cocaine. Oral administration, on the other hand, produces a more stable pharmacokinetic pattern. Nevertheless, cocaine produces euphoric effects upon oral administration. The report by Scheinin et al. (43) that IV, but not oral, administration of 100 mg of nomifensine to healthy volunteers produced euphoria in 3 of 5 subjects supports the idea that the route of administration might play an important role in determining the subjective effects of nomifensine. Unfortunately, that study reported on only five subjects, and did not provide details on how the euphoria was quantified.

Another explanation is that when given orally, these agents may not achieve a high enough concentration in the brain to inhibit DA reuptake. This hypothesis can be definitively tested using positron emission tomography and ligands which bind to the DA transporter such as [^{11}C]cocaine or [^{11}C]nomifensine. In the latter case, the fact that trace doses of [^{11}C]nomifensine bind to the DA transporter demonstrates that this drug does bind to the DA transporter in humans (3). The fact that the therapeutic effects of nomifensine, mazindol and bupropion are presumably mediated by actions in the CNS also argues against the pharmacokinetic hypothesis.

On the other hand, these drugs are given mostly to psychiatric populations, who might respond differently than a 'normal' population. Although it is true, as described by Iwamoto and Martin (24), that certain groups of people respond differently to opioid drugs, this is certainly not a documented fact for cocaine. In fact, the evidence would suggest otherwise. Cocaine is widely abused by schizophrenics (5,6), and the coexistence of cocaine abuse and other major mental disorders is now a well-documented fact (38). This is certainly not the case for nomifensine, which was widely used in nonpsychiatric patient populations to help diagnose prolactin-secreting tumors (15,36).

In addition, as generally practiced, intravenous self-administration studies in animals use a substitution procedure, in which the animal is first trained to self-administer cocaine, and the ability of a drug to substitute for the cocaine is measured. This rate-dependent approach is not the best approach for measuring the reinforcing efficacy of the drug (26). That is, the substitution method does not tell if a drug is less reinforcing than cocaine, only that the animal will self-administer it when it is denied access to cocaine. Indeed, it seems reasonable, though simplistic, to suggest that a cocaine-addicted monkey who is denied access to cocaine, will self-administer any drug which will increase synaptic DA, even if it is not as intrinsically reinforcing as cocaine. In other words, if it can't have cocaine, it'll take the next best thing.

Furthermore, as described above, the substitution procedure uses animals who have already been trained to self-administer cocaine. The reason for this is that animals learn to self-administer cocaine more rapidly than they do other drugs. Although this is an eminently practical method, its use raises two questions. One, does being exposed to cocaine alter responses to test drugs, and two, if a drug does substitute for cocaine, would a drug-naive animal also rapidly learn to self-administer the drug? Unfortunately, these questions have not been extensively investigated. However, Beardsley et al. have shown that although drug-naive rats will not self-administer MK801, rats already self-administering PCP will (4).

One interpretation of the literature reviewed above is that intravenous self-administration of a drug by animals does not necessarily predict that it will produce euphoria when orally administered to humans. The implication of this is that merely testing positive in a drug self-administration test should not preclude further preclinical and clinical evaluation of possible cocaine antagonists. It can be argued that the self-administration test probably functions as a very coarse sieve. Tests which more directly measure reinforcing efficacy in animals would clearly provide more relevant information. Indeed, it could be argued that drugs with possible therapeutic potential, such as GBR12909 (see below), would predictably substitute for cocaine in rhesus monkeys.

As pointed out in the Introduction, one strategy for developing a cocaine antagonist is to develop a drug which will bind to the DA transporter, dissociate from it very slowly, and partially inhibit the reuptake process. Such an agent would produce an insurmountable antagonism of the effects of cocaine. Moreover, the partial inhibition of DA reuptake would provide a small but stable increase in synaptic DA, not enough to produce euphoria, but enough to decrease craving for cocaine. In addition, such an agent would ideally be insoluble in aqueous solution, so that patients could not surreptitiously self-administer it intravenously. Since many of the behavioral effects of the psychomotor stimulants in animals are produced with small elevations of synaptic DA, such an ideal agent would predictably behave like a psych-

omotor stimulant. Differences might become apparent, though, in experiments designed to measure the efficacy in addition to the potency of the drug.

GBR12909 has some of the characteristics of such an ideal drug. It is almost insoluble in aqueous solution, it binds tightly to the DA transporter, it elevates ECDA, and it attenuates the ability of cocaine to elevate ECDA, which reflects inhibition of DA reuptake. It is possible that the elevation of ECDA produced by GBR12909 alone is high enough to produce euphoria in humans. In this regard, it will be important to execute structure activity studies so as to define the pharmacophore responsible for tight binding of GBR12909 to the DA transporter, the pharmacophore responsible for its ability to elevate ECDA, and the pharmacophore which defines its efficacy as a reinforcer in animals. With this information, it might be possible to develop a GBR12909-like drug which maximizes the tight binding, and minimizes the reinforcing efficacy and elevation of ECDA.

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