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STRUCTURAL REQUIREMENTS FOR COCAINE CONGENERS TO INTERACT WITH DOPAMINE AND SEROTONIN UPTAKE SITES IN MOUSE BRAIN AND TO INDUCE STEREOTYPED BEHAVIOR

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Abstract—We report here saturation analysis of [^{3}H]cocaine binding in various mouse brain regions, and the necessary structure–activity relationships for cocaine congeners to inhibit Na^+-dependent [^{3}H]-cocaine binding and [^{3}H]dopamine uptake in the mouse striatum, and to inhibit [^{3}H]cocaine binding that cannot be stimulated by Na^+ and [^{3}H]serotonin uptake in the mouse cerebral cortex. Generally similar structure–activity relationships were noted for all these processes. The ester linkage between the tropane and phenyl rings was not required for activity, in contrast to the configuration of the groups on C_2, and to a lesser extent C_3, in the tropane ring. Stereospecificity was evident from the differences between cocaine and (+)-pseudococaine, and between WIN 35,065-2 and WIN 35,065-3. There were remarkable differences between the above structure–activity relationships and those for local anesthetic activity of cocaine congeners, indicating that sodium channels were not labeled to a measurable extent with [^{3}H]-cocaine under the present conditions. Preliminary data indicated a significant correlation between the potencies of cocaine congeners in inhibiting the Na^+-dependent binding of [^{3}H]cocaine and their potencies in inducing stereotyped sniffing upon intraventricular administration.

Recent studies [1–5] described sites in the brain that saturably bind [^{3}H]cocaine with K_d values between 0.3 and 0.8 μM, in the range of brain and plasma concentrations of cocaine achieved by various routes of administration [6, 7]. These binding sites for [^{3}H]-cocaine fall into two groups. In the cerebral cortex there are sites that cannot be stimulated by Na^+ with a pharmacological profile resembling that of the recognition sites for neuronal uptake of serotonin [2]. The sites in the striatum are Na^+ dependent, and they bind inhibitors of dopamine uptake with affinities that correlate well with their potencies in blocking the neuronal uptake of dopamine [3–5]. The cerebral cortical sites are sensitive to serotonin neurotoxins such as *p*-chloroamphetamine and 5,7-dihydroxytryptamine [2], whereas the striatal sites can be destroyed by 6-hydroxydopamine [3, 5] or *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [8] or by the degeneration of the nigrostriatal pathways in Parkinson's disease [4, 5].

In a previous study we reported the potencies of five cocaine analogs in inhibiting [^{3}H]cocaine binding that "cannot be stimulated by Na^+/does not require Na^+" (COC^- binding) in the mouse cerebral cortex [9]. The present work was undertaken to obtain more detailed information on the structure–activity relationships of the ability of cocaine and its congeners to inhibit not only the COC^- binding, but also the striatal "Na^+-dependent [^{3}H]cocaine binding" (COC^+ binding). Furthermore, in this study we compare the structural requirements for cocaine congeners in inhibiting [^{3}H]cocaine binding with the requirements for inhibiting neuronal uptake of [^{3}H]serotonin in the cerebral cortex and of [^{3}H]dopamine in the striatum. We also describe results from saturation analysis of [^{3}H]cocaine binding in various brain regions. Finally, preliminary evidence is presented linking the structure–activity relationships of cocaine congeners in inhibiting [^{3}H]cocaine binding in the striatum with those in inducing stereotyped behavior.

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MATERIALS AND METHODS

Animals. For all experiments, we used adult male BALB/cBy mice weighing 19–21 g from the breeding colony of our Institute. The animals were kept on a 12-hr light/dark cycle (7:00 a.m.–7:00 p.m. light), with food and water available *ad lib.* For the behavioral studies, mice were implanted under chloral hydrate (380 mg/kg) anesthesia with 22-gauge stainless steel guide cannulas containing 28-gauge dummy cannulas, the tips of which were aimed at the third ventricle. The stereotaxic coordinates of the intended microinjection sites were 0.3 mm posterior to the bregma and 0.3 mm below the skull surface. The mice were allowed 1 week for post-surgical recovery. Cannulated mice were kept in individual cages. For microinjection in the conscious mouse, the dummy cannula was removed and an injection needle (28-gauge) was inserted. The animal was gently held in the hand with the head only slightly restrained during the insertion and removal of needles.

Binding of [^{3}H]cocaine to membrane preparations. Various anatomical regions of fresh brain were dissected and homogenized in 10 vol. of ice-cold 0.25 M sucrose in a 15-ml glass homogenizer with a motor-driven Teflon pestle (0.125-mm clearance). The homogenate was centrifuged at 1,000 *g* for 10 min at 0–4°, and the supernatant fraction was subsequently centrifuged at 17,000 *g* for 20 min. The resulting pellet (P_2) was homogenized in the ice-cold buffer indicated (always adjusted to pH 7.7 at room temperature) with a Brinkmann Polytron (setting 6, 15 sec). Portions of the P_2 preparation (0.5 ml, 0.3 to 0.6 mg protein) were incubated at 21° for 20 min with 16 nM [^{3}H]cocaine (31.1 Ci/mmole; New England Nuclear Corp., Boston, MA) and varied amounts of unlabeled cocaine or other compounds in a final volume of 0.56 ml. Binding assays were terminated on poly-L-lysine presoaked GF/B filters with a single-manifold Millipore filtration apparatus, and radioactivity was measured as described previously [2]. Nonspecific binding was defined as the residual binding observed in the presence of 30 μM unlabeled cocaine. It was equal to that defined by a 30 μM concentration of a cocaine congener, norcocaine. Under the conditions for measuring COC^- binding (see below), the nonspecific binding was 15–17% of the total binding of 16 nM [^{3}H]cocaine in the cerebellum and olfactory tubercle, and 4–12% in all other brain regions. The respective figure for striatal COC^+ binding was 3%. Binding of [^{3}H]cocaine to filters was low, and was always corrected for [2]. Under the above conditions, the binding to filters in the absence of brain membranes was approximately 0.04% of the total radioactivity in the medium. Protein was estimated by the method of Lowry *et al.* as described previously [2].

Uptake of [^{3}H]serotonin and [^{3}H]dopamine into synaptosomes. Freshly dissected parts of the brain were processed for uptake measurements as described previously [8]. Uptake of [^{3}H]serotonin binoxalate (26.7 Ci/mmole; New England Nuclear Corp.) and [^{3}H]dopamine (23.1 Ci/mmole; New England Nuclear Corp.) was terminated after 4 min by filtration through Millipore filters (0.65 μm). The final concentrations of serotonin and dopamine in the uptake assays were made 0.1 μM by addition of unlabeled compound. Neuronal uptake of [^{3}H]-serotonin was defined as total uptake minus uptake in the presence of 10 μM chlorimipramine. Neuronal uptake of [^{3}H]dopamine in the striatum was similarly defined with 50 μM benztropine.

Analysis of binding and uptake data. For the Scatchard analyses of COC^- binding, the free concentrations of [^{3}H]cocaine ranged from 15 to 1015 nM, and for the analyses of COC^+ binding from 32 to 1032 nM. A typical Scatchard analysis consisted of six points assayed in triplicate. The data were analyzed by a nonlinear curve-fitting algorithm with the LIGAND computer program developed by Munson and Rodbard [10]. Although a very slight curvature (upward-concave) was present in the Scatchard plots for the midbrain + (hypo)thalamus, hippocampus, and olfactory tubercle, two-site fits were not statistically significantly better than one-site fits when the data were analyzed by the LIGAND program.

IC_{50} Values of drugs in inhibiting the binding of [^{3}H]cocaine were estimated with a final concentration of [^{3}H]cocaine of 16 nM, well below the K_d of [^{3}H]cocaine binding, and are therefore good approximations of their actual potencies. Inhibition by four to six concentrations of each drug was assayed in triplicate in two separate tissue preparations. IC_{50} Values and their 95% confidence intervals were estimated from linear regression analysis of log-probit plots. Inhibition of monoamine uptake was analyzed in the same way.

Behavior. For intraventricular injection, the drugs were dissolved in an isotonic solution (vehicle) that was 0.12 M NaCl and 6.2 mM sodium phosphate, pH 6.7. The drugs themselves did not contribute significantly to the tonicity and did not change the pH. Compounds that came as free bases were first dissolved in 25 mM sodium phosphate buffer, pH 6.7; 0.15 M NaCl was added to obtain the above concentrations. Immediately after receiving an intraventricular injection of 3 μl, the animal was placed in a plastic cage that was, like his home cage, 27 × 17 × 12 cm, and whose floor was covered with a layer of shavings. The animal was then observed by one of the authors (M.E.A.R.) for a period of 10 min. The total duration of sniffing and biting in that period was quantified with timers. All drugs shown in Fig. 1 produced dose-dependent increases in the durations of sniffing and of biting skin or paws; they did not produce licking. The sniffing also included sustained forward head searching movements, which were more prominent at the higher drug doses; in the latter case, the sniffing episodes often lasted more than 15 sec. Episodes of biting, though vigorous, usually lasted less than 15 sec. In our animals, sniffing was the major activity at all doses studied. We therefore used only the sniffing times as the basis for estimating the stimulation thresholds (see below). The behavioral measurements were made in a quiet room separate from the laboratory space used for the biochemical work. Room temperature was kept at 22–24° by a constantly humming window air conditioner. Various doses of each drug were tested, 0.01 mg/kg and up with 2-fold differences between doses. The sniffing stimulation threshold represents the minimal dose of a drug to augment the duration of sniffing of an animal as compared with the duration after vehicle alone in that animal, measured in at least two separate animals. Over a 2-week period after postsurgical recovery, animals were injected more than once (at least one day apart) with different drugs in different sequences. On the average, ten behavioral sessions were held for establishing the stimulation threshold for a particular drug involving five different drug doses and seven separate animals. Replicates of vehicle alone in the same animal and between animals were within a close range (95 ± 6 sec/10 min, average ± S.E.M. for twenty-eight animals). We are aware of reports of sensitization to certain effects of cocaine in the rat (see Results) upon prolonged treatment, but we have not found evidence for this phenomenon in the mouse under the conditions of our experiments (different drugs in different sequences in the same animal). A number of animals lost the cannula before the 2-week test period was

over; these animals were included in the analysis. Whenever possible, correct placement of the cannulas was verified by injecting Evans blue prior to killing the animal.

Materials. The following persons or companies donated the drugs indicated: Dr. S. B. Ross, Astra (Sweden), benzoyltropine and benzoylpseudotropine; Dr. K. A. Nieforth (Storrs, CT), *N*-allylnorcocaine hydrochloride; Merck (Darmstadt, Germany), (+)-pseudococaine and (+)-neopseudococaine hydrogen tartrate; Sterling-Winthrop Research Institute (Rensselaer, NY), WIN 35,428, WIN 35,065-2, WIN 35,140, WIN 35,004, and WIN 35,065-3; and National Institute on Drug Abuse, Research Triangle Institute (Research Triangle Park, NC), norcocaine. Mallinckrodt Chemical Corp. (St. Louis, MO) was the source of cocaine hydrochloride.

RESULTS

[^{3}H]Cocaine binding in various brain regions and its relationship to neuronal uptake of monoamines. Previously [11] we found that 25–50 mM Na^+ stimulated [^{3}H]cocaine binding markedly in the striatum (4-fold), somewhat in the olfactory tubercle (1.3-fold), and not at all in other mouse brain regions. These Na^+ concentrations did not inhibit binding in any region other than the cerebellum [11, 12]. Higher concentrations of Na^+ (100–200 mM) were inhibitory in all brain regions [11]. We will therefore, in the following, distinguish Na^+-dependent [^{3}H]cocaine (COC^+) binding, abundant in the striatum and present to some extent in the olfactory tubercle, and [^{3}H]cocaine binding that cannot be stimulated by Na^+/does not require Na^+ (COC^- binding), present in all brain regions. In the present study, for the measurement of COC^- binding in the striatum and olfactory tubercle, we used potassium phosphate buffer at a very low concentration, 5 mM, since Kennedy and Hanbauer [3] have shown that high concentrations of K^+ are inhibitory. Control of pH at such low buffer concentrations is more difficult, and for that reason we chose not to use 5 mM potassium phosphate as the routine condition for assaying COC^- binding in brain regions other than the striatum or olfactory tubercle; instead we used 25 mM sodium phosphate buffer, which contains approximately 48 mM Na^+ from the primary and secondary phosphate salts mixed to pH 7.7 at room temperature, and which therefore does not inhibit or stimulate COC^- binding.

The results from weighted least-squares nonlinear curve-fitting with the LIGAND computer program [10] are shown in Table 1. The difference in B_{max} between binding in the presence and absence of 48 mM Na^+ was much smaller for the olfactory tubercle than for the striatum. This difference for the striatum is such that the contribution of COC^- binding to the binding measured in the presence of 48 mM Na^+ in the striatum is in the order of 25%. Therefore, the bulk of striatal binding in the presence of 48 mM Na^+ is COC^+ binding. In the olfactory tubercle, the bulk of the binding is COC^- binding. In all other brain regions, the binding measured in the presence of 48 mM Na^+ is only COC^- binding,

Table 1. Binding of [^{3}H]cocaine and uptake of [^{3}H]serotonin in regions of mouse brain

Region	[^{3}H]Cocaine binding*			[^{3}H]Serotonin uptake
	K_d (nM)	B_{max} (pmoles/mg protein)	At 16 nM†	Velocity (pmoles/mg protein/min)
	COC^- binding‡			
Cerebral cortex	149 ± 10	1.23 ± 0.12	0.128 ± 0.012	1.22 ± 0.16
Midbrain + (hypo)thalamus	154 ± 28	1.67 ± 0.21	0.195 ± 0.026	2.28 ± 0.15
Hippocampus	188 ± 21	1.09 ± 0.05	0.096 ± 0.015	0.99 ± 0.23
Pons/medulla	175 ± 12	2.05 ± 0.41	0.184 ± 0.026	1.84 ± 0.03
Cerebellum	396 ± 74	1.05 ± 0.25	0.044 ± 0.005	0.20 ± 0.08
Olfactory tubercle§	240 ± 52	1.70 ± 0.14	0.110 ± 0.017	1.49 ± 0.08
Striatum§	338 ± 23	1.94 ± 0.24	0.095 ± 0.016	1.20 ± 0.08
	COC^- plus COC^+ binding‡			
Olfactory tubercle	153 ± 8	2.43 ± 0.38	0.203 ± 0.031	
Striatum	122 ± 14	7.12 ± 0.34	0.651 ± 0.096	

Results are means and their ranges of data obtained with two independent membrane preparations assayed in triplicate. Each preparation was a pool of tissue from a number of mice, ranging from fifteen for the striatum (the smallest area) to four for the cerebral cortex (the greatest area).

* Binding data were obtained and analyzed with the LIGAND program as described under Materials and Methods. All assays, except for results indicated by §, were performed in the presence of 25 mM sodium phosphate buffer.

† Concentration of free [^{3}H]cocaine.

‡ COC^- binding, [^{3}H]cocaine binding that cannot be stimulated by Na^+/does not require Na^+; COC^+ binding, Na^+-dependent [^{3}H]cocaine binding (see Results).

§ Cocaine binding was assayed with 5 mM potassium phosphate buffer.

since (i) 48 mM Na^+ had no stimulatory or inhibitory effect [11, 12], and (ii) saturation analysis of [^{3}H]-cocaine binding in 5 mM potassium phosphate buffer indicated essentially the same B_{max} values as measured in 25 mM sodium phosphate buffer (data not shown). In all regions except the cerebellum, the binding exhibited K_d values between 122 and 188 nM when assayed with 25 mM phosphate buffer (Table 1). The slightly higher K_d values for the binding in the absence of Na^+ in the olfactory tubercle and striatum are probably due to the use of 5 mM potassium phosphate buffer, since saturation analysis of [^{3}H]cocaine binding in that buffer indicated K_d values averaging 286 nM (data not shown) as compared with 166 nM (Table 1) in the presence of 25 mM sodium phosphate in the cerebral cortex, midbrain + (hypo)thalamus, hippocampus, and pons-medulla. The rather high K_d for the cerebellum may indicate the presence of a different population of cocaine binding sites.

There was a significant correlation ($r = 0.95$; $N = 7$; $P < 0.002$) between COC^- binding at a fixed, low concentration of 16 nM and the neuronal uptake of [^{3}H]serotonin in various brain regions (Table 1). Although the correlation between the B_{max} of COC^- binding and [^{3}H]serotonin uptake in the regions was statistically positive ($r = 0.75$; $N = 7$; $P = 0.05$) (Table 1), it was weak with exceptional areas such as the cerebellum and striatum. There was no relationship among brain regions (data not shown) between COC^- binding and uptake of [^{3}H]dopamine into noradrenergic and dopaminergic nerve terminals as estimated by the use of 1 μM desipramine to selectively block the uptake of [^{3}H]dopamine into noradrenergic terminals.

Structure–activity relationship for the ability of cocaine and its congeners to inhibit [^{3}H]cocaine binding, [^{3}H]serotonin uptake, and [^{3}H]dopamine uptake. COC^- binding in the cerebral cortex was inhibited by cocaine congeners with a wide range of potencies: there was a 500-fold difference between the most potent substance, WIN 35,428, and the weakest, WIN 35,065-3 (Table 2). Stereospecificity was evident from the differences between cocaine and (+)-pseudococaine, and between WIN 35,065-2 and WIN 35,065-3. Compounds such as benzoylecgonine and ecgonine had IC_{50} values of more than 1 mM, the highest concentration tested (data not shown). A generally similar rank order of IC_{50} values was observed for inhibition of COC^+ binding in the striatum, although there were also some marked differences. WIN 35,428 and WIN 35,065-2 were relatively more potent in the striatum, whereas (+)-neopseudococaine and benzoyltropine were weaker (Table 2). Most substances inhibited binding with Hill numbers close to unity.

There was good correlation between the IC_{50} values of cocaine congeners for the inhibition of the neuronal uptake of [^{3}H]serotonin in the cerebral cortex and those for the inhibition of COC^- binding in that region (Table 2) ($r = 0.95$; $N = 12$; $P < 0.001$), indicating that the structural requirements for inhibition of neuronal uptake of serotonin by cocaine are the same as those for inhibition of COC^- binding. Likewise, there was a highly significant correlation between the values of cocaine congeners in inhibiting the striatal uptake of [^{3}H]-dopamine and in inhibiting the striatal COC^+ binding ($r = 0.98$; $N = 12$; $P < 0.001$). The same compounds (WIN 35,428 and WIN 35,065-2) that were more potent on [^{3}H]cocaine binding in the striatum than in the cortex were also more potent in inhibiting striatal dopamine uptake than cerebrocortical serotonin uptake; conversely, drugs such as (+)-neopseudococaine and benzoyltropine were weaker in the striatum than in the cortex (Table 2). In the cerebral cortex, serotonin was two orders of magnitude weaker in inhibiting COC^- binding than in inhibiting serotonin uptake, and in the striatum dopamine was similarly weaker in inhibiting COC^+ binding than in inhibiting dopamine uptake (Table 2).

Relationship between binding of cocaine/cocaine congeners and behavior. Stimulation of dopamine receptors in the striatum is likely to be involved in the production of stereotyped sniffing, licking, biting, and gnawing in the rat by amphetamine (for a recent review, see Beninger [13]). Since cocaine is a potent blocker of striatal dopamine uptake and also can produce stereotypies, especially upon repeated administration [14–16], we made an attempt at measuring stereotyped behavior in male BALB/cBy mice as used in the above biochemical experiments, and relating this to the binding of cocaine congeners to striatal membranes. It has been reported that the intensity and qualitative nature of the stereotypies following acute cocaine differ from those following amphetamine [17, 18]. Instead of the stereotypy rating scales developed for the effects of amphetamine in the rat, we measured the total duration of sniffing and biting behavior during a full 10-min period after intraventricular injection of cocaine or a cocaine congener. On the average for all drugs, we found an increase of 74% in the total duration of sniffing at the stimulation threshold as compared with that after vehicle alone. For every drug, there was a statistically significant difference ($P < 0.05$, two-tailed Mann-Whitney U test) between the sniffing times at doses below stimulation threshold and those at doses at and above stimulation threshold.

There was a significant correlation ($r = 0.84$; $N = 11$; $P < 0.002$) between the potencies of cocaine and of cocaine congeners in inhibiting COC^+ binding in the striatum and the sniffing stimulation thresholds (Fig. 1).

DISCUSSION

A certain pattern of selectivity for cocaine congeners was shared by COC^- and COC^+ binding sites, the neuronal serotonin uptake complex, and the neuronal dopamine uptake complex. Removal of the ester linkage between the tropane and phenyl rings (WIN 35,428 and WIN 35,065-2, Table 2) did not reduce the affinity of the molecule for cocaine binding sites or monoamine uptake sites. Moving the carbomethoxy group (R_2) on C_2 from an axial (cocaine and WIN 35,065-2) to an equatorial ((+)-pseudococaine and WIN 35,140) position clearly diminished the potency. Likewise, moving the *O*-benzoyl group (R_4) on C_3 from an equatorial (ben-

Table 2. Inhibition of [^{3}H]cocaine binding, [^{3}H]serotonin uptake, and [^{3}H]dopamine uptake by cocaine congeners and monoamines

	Group substituents					$-\log IC_{50}$*			
						Cortex		Striatum	
	R_1	R_2	R_3	R_4	R_5	COC binding†	5-HT uptake†	COC binding†	DA uptake†
1. Cocaine	CH_3	CO_2CH_3	H	OOCPh‡	H	6.78 ± 0.11§	5.75 ± 0.26	6.86 ± 0.06	6.06 ± 0.16
2. WIN 35,428	CH_3	CO_2CH_3	H	PhF‖	H	7.02 ± 0.15	6.24 ± 0.19	7.57 ± 0.15	7.10 ± 0.10
3. WIN 35,065-2	CH_3	CO_2CH_3	H	Ph	H	6.64 ± 0.18	5.89 ± 0.15	7.39 ± 0.11	6.73 ± 0.19
4. WIN 35,140	CH_3	H	CO_2CH_3	Ph	H	5.17 ± 0.12	3.61 ± 0.21	5.48 ± 0.10	5.26 ± 0.20
5. WIN 35,004¶	CH_3	H	CH_2OAc	Ph	H	5.15 ± 0.18	4.04 ± 0.40	5.41 ± 0.06	5.08 ± 0.09
6. WIN 35,065-3**	CH_3	CO_2CH_3	H	Ph	H	4.32 ± 0.41	3.55 ± 0.23	4.49 ± 0.32	3.70 ± 0.24
7. Norcocaine	H	CO_2CH_3	H	OOCPh	H	6.92 ± 0.27	6.76 ± 0.20	6.57 ± 0.08	5.62 ± 0.22
8. *N*-Allylnorcocaine	CH_2 | $HCCH_2$	CO_2CH_3	H	OOCPh	H	6.05 ± 0.15	5.31 ± 0.38	5.99 ± 0.10	5.60 ± 0.06
9. (+)-Pseudococaine	CH_3	H	CO_2CH_3	OOCPh	H	5.48 ± 0.14	4.56 ± 0.24	5.07 ± 0.30	4.44 ± 0.05
10. (+)-Neopseudococaine	CH_3	H	$CO_2C_3H_7$	OOCPh	H	6.14 ± 0.19	5.67 ± 0.21	5.33 ± 0.24	4.75 ± 0.08
11. Benzoylpseudotropine	CH_3	H	H	OOCPh	H	5.75 ± 0.04	4.92 ± 0.12	5.48 ± 0.07	4.73 ± 0.05
12. Benzoyltropine	CH_3	H	H	H	OO | PhC	5.16 ± 0.33	4.58 ± 0.23	4.64 ± 0.18	3.96 ± 0.26
13. Serotonin						4.38 ± 0.19	6.76 ± 0.52		
14. Dopamine								4.70 ± 0.15	6.27 ± 0.23

* IC_{50} Values were estimated by linear regression analysis of log-probit plots as described under Materials and Methods.

† Binding of cocaine (COC) to membranes was assayed with 25 mM sodium phosphate buffer. Synaptosomal preparations were used for the measurement of uptake of serotonin (5-HT) and dopamine (DA).

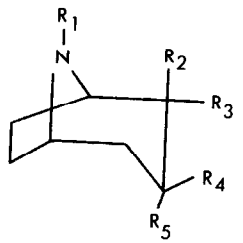
‡ Ph = phenyl.

§ 95% Confidence limits.

‖ PhF = phenyl with F in the para position.

¶ Ethylene bridge between C_1 and C_5 in tropane ring is removed.

** Compound 6 is enantiomer of 3.



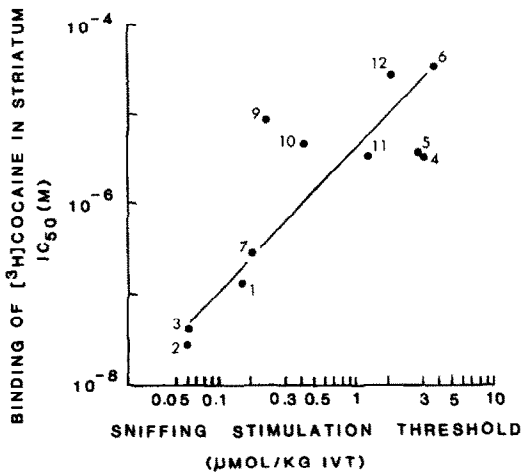


Fig. 1. Drug potencies in inhibiting *in vitro* the Na^+-dependent [^{3}H]cocaine binding in the striatum, and in producing *in vivo* sniffing after intraventricular injection. The sniffing stimulation thresholds were determined as described in Materials and Methods and in Results. The numbering of the compounds is as in Table 2.

zoylpseudotropine) to an axial (benzoyltropine) position reduced the activity of the molecule. The position of the C_2 constituent seems to be more important than that of the C_3 constituent, since the difference in potency between WIN 35,065-2 and WIN 35,140 was greater than between benzoylpseudotropine and benzoyltropine; the difference between cocaine and (+)-pseudococaine was also greater than between benzoylpseudotropine and benzoyltropine. Removal of the C_2 constituent (benzoylpseudotropine versus cocaine) appreciably reduced the activity, but had a less profound effect than removal of the methylester from the C_2 position of cocaine (benzoylecgonine), which rendered the molecule inactive (data not shown). It is possible that this was due to the strongly reduced lipophilicity of the highly polar benzoylecgonine; ecgonine itself was also inactive (data not shown). WIN 35,065-3 is a pure enantiomer of WIN 35,065-2; the two compounds are *d*- and *l*-isomers, respectively, of the phenyltropane analog of cocaine [19]. WIN 35,065-3 was between two and three orders of magnitude weaker than WIN 35,065-2, indicating a definite stereospecificity of the cocaine binding sites and monoamine uptake sites. The above structure–activity relationships are notably different from those for local anesthetic activity of cocaine congeners: (1) removal of the ester linkage between the tropane and phenyl rings drastically reduces local anesthetic potency [19], (2) placing the R_2 group in an equatorial position enhances local anesthetic activity and interaction with sodium channels [20], and (3) the position of the C_2 constituent is less important than that of the C_3 constituent for interaction with the sodium channel [20], indicating that local anesthetic properties are unimportant with regard to central effects on monoamines.

There are some interesting differences between the cerebral cortex and the striatum in the interactions of cocaine congeners with cocaine binding sites and monoamine uptake sites. Removal of the ester linkage between the tropane and phenyl rings made the molecule more potent in the striatum, whereas N-demethylation (norcocaine) resulted in some loss of activity in the striatum but not in the cerebral cortex (Table 2). Extension of the carbomethoxy group in (+)-pseudococaine (yielding (+)-neopseudococaine) had a greater effect in increasing the potency in the cerebral cortex than in the striatum; C_3 epimerization of benzoylpseudotropine (yielding benzoyltropine) had a greater effect in reducing the potency in the striatum than in the cerebral cortex. Such differences may indicate that the cocaine and cocaine congeners interacted at basically different sites on the serotonin transporter in the cerebral cortex and on the dopamine transporter in the striatum. Alternatively, cocaine and cocaine congeners may have interacted with a protein involved in co- or countertransport of ions, a protein that is shared by the neuronal uptake system for serotonin and that for dopamine. The above differences would then be due to different environments of this protein in serotonergic and dopaminergic nerve terminals. No satisfactory explanation can be offered at this time for the apparent lack of relation between cocaine binding and the uptake of norepinepherine, especially since cocaine is a good blocker of the neuronal uptake of norepinephrine.

It is of interest that the structural requirements for cocaine congeners in inhibiting the monoamine uptake mechanism were the same as those for inhibiting the binding of [^{3}H]cocaine to sites associated with these uptake systems. Not only the rank order of potencies, but also their absolute values, were comparable (Table 2). The somewhat higher IC_{50} values for uptake inhibition were most likely due to the fact that the difference between the concentration of radioactive monoamine (0.1 μM) in the uptake assays and its K_m for transport into synaptosomes (0.1 to 0.2 μM) was smaller than the difference between the concentration of [^{3}H]cocaine in the binding assays (16 nM) and its K_d (120–150 nM). The comparable absolute values of potency in uptake and binding are consonant with the proposal that [^{3}H]cocaine labels presynaptic uptake regulatory sites at which cocaine and cocaine congeners act to inhibit neuronal uptake of monoamines. It should be emphasized, however, that the monoamines themselves were much weaker in inhibiting [^{3}H]-cocaine binding than in inhibiting monoamine uptake (Table 2). This is in agreement with other results, and possible explanations have been discussed elsewhere [3, 5].

The pharmacological relevance of the COC^- binding sites is a matter of speculation. They seem to be associated with neuronal uptake of serotonin, even in the striatum, where COC^- binding has a pharmacological profile resembling that of serotonin uptake sites and where COC^- binding can be reduced by 5,7-dihydroxytryptamine lesioning [21]. Serotonin is involved in numerous processes (for a review see Ref. 22), and interference with its neuronal uptake via cocaine binding sites could perhaps contribute to the anorexic, locomotor, euphoric, or sleep-disruptive effect of cocaine. COC^+ binding in the striatum is related to the neuronal uptake of

dopamine. The correlation between the inhibition of striatal binding by cocaine congeners and the induction of stereotyped sniffing in preliminary experiments (Fig. 1) is in agreement with the notion that the COC^+ binding sites in the striatum are involved in cocaine-induced increases in stereotyped elements of behavior; this, in turn, fits in with the implication of striatal dopamine in the generation of such behavior. Although the behavioral data presented here have a preliminary character, the drug effects were consistent and very impressive at the higher doses. It is worth mentioning here that intracerebral administration of cocaine congeners was a necessary procedure for measuring their central effects, because systemic administration reveals mainly their local anesthetic effects in inhibiting the locomotor activity of the mice [23, 24]. Undoubtedly, appreciable caution is necessary in interpreting the effects of *in vivo* drugs that have to be transported to their presumed sites of action, that are susceptible in the meantime to metabolizing enzymes, and that may have multiple effects. Some of these variables may be minimized by limiting study to a set of closely related chemical structures, and by applying the drugs close to the presumed site of action. Even with these reservations, the preliminary behavioral results are of considerable interest. It has been reported that chronic administration of cocaine increases its effects on stereotyped behavior [14, 16]; the increase in the B_{max} of COC^+ binding in the rat striatum observed by Missale *et al.* [25] is consonant with a role of these sites in the generation of stereotyped behavior, as indicated also by the present work.

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