0026-895X/95/040779-08$3.00/0

MOLECULAR PHARMACOLOGY, 47:779–786 (1995).

Selective Labeling of the Dopamine Transporter by the High Affinity Ligand 3β-(4-[^{125}I]Iodophenyl)tropane-2β-carboxylic Acid Isopropyl Ester

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Received August 8, 1994; Accepted December 14, 1994

SUMMARY

The iodine-125 analog of the dopaminergically selective cocaine analog 3β-(4-iodophenyl)tropane-2β-carboxylic acid isopropyl ester (RTI-121) was evaluated as a probe for the dopamine transporter in rat striatum. Saturation and kinetic studies indicated that [^{125}I]RTI-121 binds to both high and low affinity components. The K_d of the high affinity component was 0.14 ± 0.01 nM (mean ± standard error), whereas the low affinity component demonstrated an affinity of 1.59 ± 0.09 nM. The corresponding numbers of striatal binding sites labeled by [^{125}I]RTI-121 were 295 ± 6 and 472 ± 59 pmol/g of tissue (original wet weight), respectively. Intrastriatal injections of 6-hydroxydopamine eliminated >90% of specific [^{125}I]RTI-121 binding in the striatum. The pharmacological profile of specific [^{125}I]RTI-121 binding in the rat striatum was consistent with that of the dopamine transporter. There was a strong ($r = 0.98$, $p < 0.0001$) correlation between the potencies of drugs that displaced specific [^{125}I]RTI-121 binding and the potencies of these drugs to inhibit the uptake of [^{3}H]dopamine. In contrast, no correlation was found for the potencies of drugs to inhibit the uptake of either [^{3}H]norepinephrine or [^{3}H]serotonin. Autoradiographs produced using [^{125}I]RTI-121 demonstrated a distribution of label consistent with the distribution of dopaminergic neurons in rat brain. Because of its high affinity and high selectivity for the dopamine transporter, [^{125}I]RTI-121 may be an extremely useful ligand for the dopamine transporter.

Several studies have identified specific binding sites for cocaine and its analogs in rodent, nonhuman primate, and human brain (1–11). These binding sites demonstrate several properties that are associated with a biologically relevant receptor, including stereoselectivity (7, 12), binding at biologically relevant concentrations (13, 14), a significant correlation between binding affinities and uptake potencies (3, 7, 8, 9, 11), and a correlation between binding potency and potency to maintain drug self-administration behavior (15, 16). Thus, the evidence suggests that cocaine initiates its biological action via interaction with a relevant receptor located on the DAT.

As early as 1980, cocaine was recognized as a potential ligand for the DAT. However, several problems were apparent with the use of cocaine as a radioligand. These included low affinity combined with rapid dissociation and poor selectivity for the DAT. Several alternative ligands, which are all inhibitors of DA uptake, have been developed to probe the DAT. These compounds can be divided into two groups, those that, like cocaine, bind to both high and low affinity components and those that bind to a single, high affinity component. The latter group, which includes [^{3}H]mazindol (17), [^{3}H]nomifensine (18), [^{3}H]methylphenidate (19), and [^{3}H]GBR 12935 (20), address the problem of low affinity; however, because they bind only to a high affinity component, the site to which they bind may or may not be identical to the site recognized by cocaine (6–8, 17–25). Moreover, these ligands also recognize sites other than that associated with the DAT; for example, mazindol and nomifensine bind to the NE transporter (17, 18), and GBR 12935 binds to cytochrome P-450 or a related protein (20–22, 24, 25).

Ligands that bind to both the high and low affinity components recognized by cocaine include [^{3}H]cocaine (1–7) and the cocaine analogs [^{3}H]WIN 35,428 (8), [^{3}H]WIN 35,065–2

This research was supported in part by National Institutes of Health Grant DA05477.

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ABBREVIATIONS: DAT, dopamine transporter; DA, dopamine; NE, norepinephrine; RTI-121, 3β-(4-iodophenyl)tropane-2β-carboxylic acid isopropyl ester; GBR 12909, 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride; GBR 12935, 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride; WIN 35,428, 3β-(4-fluorophenyl)tropane-2β-carboxylic acid methyl ester; RTI-55, 3β-(4-iodophenyl)tropane-2β-carboxylic acid methyl ester; 5-HT, 5-hydroxytryptamine; 6-OHDA, 6-hydroxydopamine.

(9), and [^{125}I]RTI-55 (10, 11). Although some of the problems associated with the single-site ligands were partially eliminated by the use of either [^{3}H]WIN 35,428 or [^{3}H]WIN 35,065–2, these ligands were problematic for autoradiographic studies because they are radiolabeled with tritium and thus present problems of quenching and require long exposure times. As part of an ongoing research effort to characterize the cocaine binding site located on the DAT, [^{125}I]RTI-55 was developed as a high affinity iodinated ligand for the DAT (10, 11). However, the use of this ligand also became problematic because of its high affinity for the serotonin (5-HT) transporter as well as the DAT (11, 26–29). It was therefore important to develop cocaine analogs that would bind to the DAT with high affinity and selectivity, relative to the NE and 5-HT transporters.

Recently, we identified a new, high affinity, iodinated ligand selective for the DAT, relative to the 5-HT or NE transporters, in both *in vitro* (30, 31) and *in vivo* binding studies (32). The present study describes the *in vitro* binding of [^{125}I]RTI-121 to rat striatal membranes. The results of this study suggest that [^{125}I]RTI-121 may be the most selective, high affinity, iodinated ligand presently available for the DAT.

Materials and Methods

Tissue preparation. Adult male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 200–250 g were killed by decapitation. The striata of the rats were quickly dissected, frozen on a block of dry ice, and stored at −70° until needed.

[^{125}I]RTI-121 binding. All tissues were weighed and homogenized with a Polytron homogenizer (setting 5–6) for 15 sec, in 20 volumes of ice-cold 10 mM phosphate buffer, pH 7.4, containing 0.32 M sucrose. The homogenate was centrifuged for 10 min at 50,000 × *g*. The resulting pellet was resuspended in the aforementioned buffer to a tissue concentration of 1 mg (original wet weight)/ml, using a Polytron homogenizer (5–10 sec), and was recentrifuged; the resulting pellet was then resuspended and used in the binding assays. Binding assays, in a total volume of 2 ml, were conducted in assay tubes containing 15 pM [^{125}I]RTI-121 and 0.1 mg of striatal tissue. The nonspecific binding of [^{125}I]RTI-121 was defined by the addition of 50 μM (−)-cocaine. Striatal suspensions were incubated for 1 hr at 22–24°. The incubation was terminated by rapid filtration through Whatman GF/B filters (which had been previously soaked in 0.05% polyethylenimine), using a Brandel R48 filtering manifold (Brandel Instruments, Gaithersburg, MD). The filters were washed three times with 7 ml of ice-cold buffer and counted using an LKB-Wallac CliniGamma 1272 γ counter (LKB-Wallac, Turku, Finland).

Saturation of [^{125}I]RTI-121 binding. Assays were carried out under the conditions described above, using a fixed concentration of [^{125}I]RTI-121 (15 pM) and increasing concentrations of unlabeled RTI-121 (1 pM to 100 nM). Due to the limited amount of [^{125}I]RTI-121 available, saturation experiments using increasing concentrations of [^{125}I]RTI-121 could not be conducted.

Intrastriatal 6-OHDA lesions. Intrastriatal 6-OHDA lesions of the nigrostriatal DA pathways were performed as described previously (33). Briefly, male Sprague-Dawley rats were given an intraperitoneal injection of chloral hydrate (400 mg/kg) and then placed into a stereotaxic instrument. The animals were given injections of 20 μg of 6-OHDA in 2 μl of saline containing 0.02% ascorbic acid, at two sites of the left striatum (coordinates, AP 1.6, ML 2.4, DV 4.2 and AP 0.2, ML 2.6, DV 7.0, from Bregma). The 6-OHDA was infused at a rate of 1 μl/min through a Hamilton syringe. At the end of the infusion, the needle was left in place for another 5 min, to allow for diffusion away from the site of injection.

Autoradiographic studies using [^{125}I]RTI-121. Tissue for the visualization of [^{125}I]RTI-121 binding was obtained from male Sprague-Dawley rats according to the general method of Kuhar and Unnerstall (34). The animals were decapitated, and the brains were immediately removed and frozen in isopentane (−50°). The frozen brains were sectioned to 10-μm thickness, thaw-mounted onto gelatin-coated slides, dried, and stored desiccated at −70° until used for autoradiography. Sections were equilibrated at room temperature and incubated for 60 min at 22–24° with 15 pM concentrations of either [^{125}I]RTI-121 or [^{125}I]RTI-55, in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10.14 mM Na_2PHO_4, 1.76 mM KH_2PO_4),[2] in quadruplicate. To reduce nonspecific binding, 10 mM NaI was added to the assay buffer. The tissue sections were washed twice (for 20 min each time) in the assay buffer (0°), followed by a dip in deionized water (also at 0°). Nonspecific binding of [^{125}I]RTI-121 was defined by the addition of 50 μM (−)-cocaine. The slide-mounted sections were immediately dried with a stream of cold dry air. The dry labeled slides and ^{125}I-methylmethacrylate autoradiographic standards (Amersham, Arlington Heights, IL) were coexposed to Hyperfilm ^{3}H (Amersham) for 2–4 days.

Materials. Unlabeled RTI-121 was synthesized as described previously (30); the [^{125}I]RTI-121 precursor 3β-(4′-trimethyltinphenyl)-2β-carboxylic acid isopropyl ester and [^{125}I]RTI-121 were prepared as described for [^{123}I]RTI-55 (35).[3] [^{125}I]RTI-121 and [^{125}I]RTI-55 were also prepared by New England Nuclear, which generously supplied them as gifts. The following drugs were used in this study: amitriptyline HCl, Research Biochemicals (Natick, MA); benztropine mesylate, Research Biochemicals; citalopram HCl, H. Lundbeck A/S (Copenhagen, Denmark); (−)-cocaine HCl, National Institute on Drug Abuse (Rockville, MD); chloripramine HCl, Research Biochemicals; (+)-cocaine free base, National Institute on Drug Abuse; desipramine HCl, Sigma Chemical Co. (St. Louis, MO); GBR 12909, Research Biochemicals; GBR 12935, Research Biochemicals; 6-OHDA HCl, Sigma; imipramine HCl, Research Biochemicals; mazindol, Sandoz Pharmaceuticals (East Hanover, NJ); nisoxetine HCl, Eli Lilly Co. (Indianapolis, IN); nomifensine maleate, Hoechst-Roussel Pharmaceuticals (Somerville, NJ); nortriptyline HCl, Research Biochemicals; paroxetine HCl, Beecham Pharmaceuticals Research Division (Philadelphia, PA); talsupram HCl, Lundbeck; tomoxetine HCl, Eli Lilly Co.; WIN 35,428, National Institute on Drug Abuse.

Results

Kinetics of specific [^{125}I]RTI-121 binding. The specific binding of [^{125}I]RTI-121 reached equilibrium in approximately 40–50 min at 22–24° (Fig. 1, *top*). Analysis of the data using the computer program KINETIC revealed that [^{125}I]RTI-121 association occurred in two phases, with the first phase having a $t_{1/2}$ of 0.89 ± 0.26 min (mean ± standard deviation, two experiments) and the second phase a $t_{1/2}$ of 5.84 ± 0.65 min. Likewise, the dissociation of specifically bound [^{125}I]RTI-121 occurred in two phases (Fig. 1, *bottom*), with corresponding $t_{1/2}$ values of 3.07 ± 1.07 and 11.5 ± 0.52 min. A biexponential model for both the association and dissociation of specifically bound [^{125}I]RTI-121 was preferred over the monoexponential model in all cases ($p < 0.05$).

Saturation analysis of specific [^{125}I]RTI-121 binding. The affinity and density of specific [^{125}I]RTI-121 binding sites was determined using a fixed concentration of labeled RTI-121 (15 pM) and increasing concentrations of unlabeled RTI-121 (1 pM to 100 nM) (Fig. 2). Scatchard transformation of the data (Fig. 2, *inset*) resulted in a curvilinear relation-

[2] R. Lew, personal communication.
[3] J. R. Lever, unpublished observations.

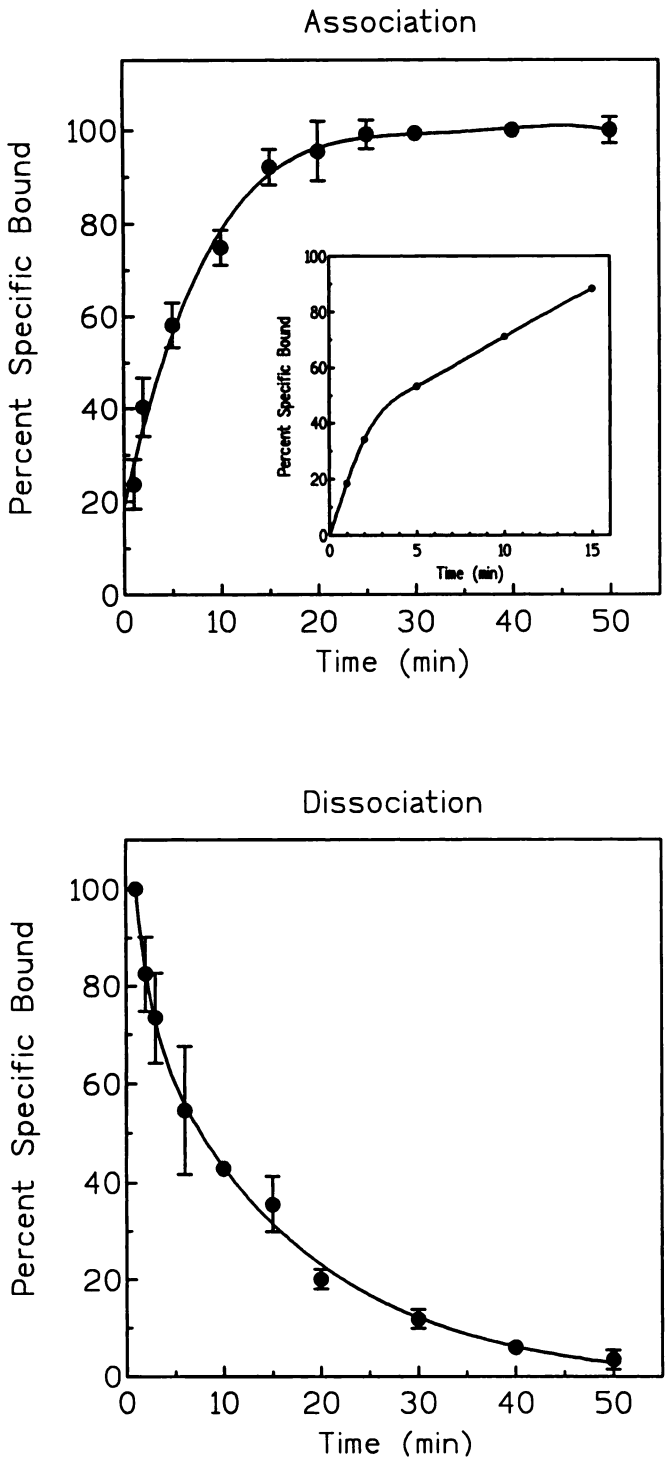

Fig. 1. Association (*top*) and dissociation (*bottom*) of [125I]RTI-121 with rat striatal membranes. Association of 15 pM [125I]RTI-121 was determined by incubating striatal membranes (0.1 mg/assay tube) at 22–24° for various times. *Inset*, the first 15 min of one experiment on an expanded scale, to more clearly show the two phases of association. The dissociation rate was determined (after equilibrium had been reached) by the addition of 1 μM unlabeled RTI-121 and termination of the reaction by rapid filtration at various times. Data from both the association and dissociation experiments were fitted by the KINETIC program to a biexponential model. The results are the means of two experiments, with triplicate determinations.

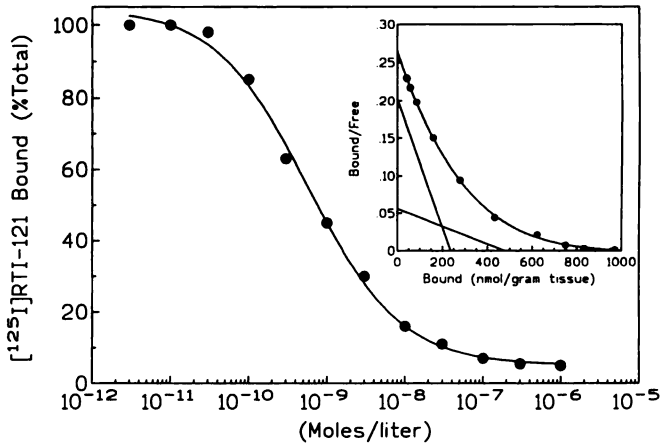

Fig. 2. Saturation of specific [125I]RTI-121 binding in rat striatum. Striatal membranes were incubated with a fixed concentration of [125I]RTI-121 (15 pM) and increasing concentrations of unlabeled RTI-121 (1 pM to 100 nM). Nonspecific binding was determined by the addition of 50 μM (−)-cocaine. The results are the mean of three independent experiments, each performed in triplicate. The fit of this plot was determined using EBDA. *Inset*, Scatchard transformation of the resulting data.

ship, suggesting a two-component model for the data; this two-component model was statistically preferred over a single-site model in all three experiments ($p < 0.01$, F test). Analysis of the data revealed a high affinity component with an affinity of 0.14 ± 0.01 nM (mean ± standard error) and a low affinity component with an affinity of 1.59 ± 0.09 nM. The numbers of corresponding striatal sites labeled by [125I]RTI-121 were 295 ± 6 and 472 ± 59 pmol/g of tissue (original wet weight), respectively. Intrastriatal injections of 6-OHDA resulted in >90% loss of specific [125I]RTI-121 binding in striatal membranes (Fig. 3). Analysis of saturation experiments conducted on tissue pretreated with 6-OHDA indicated that this reduction was a combination of a complete loss of the high affinity binding component and a partial loss of the low affinity component.

Similar results were obtained with CD-1 mouse striatum

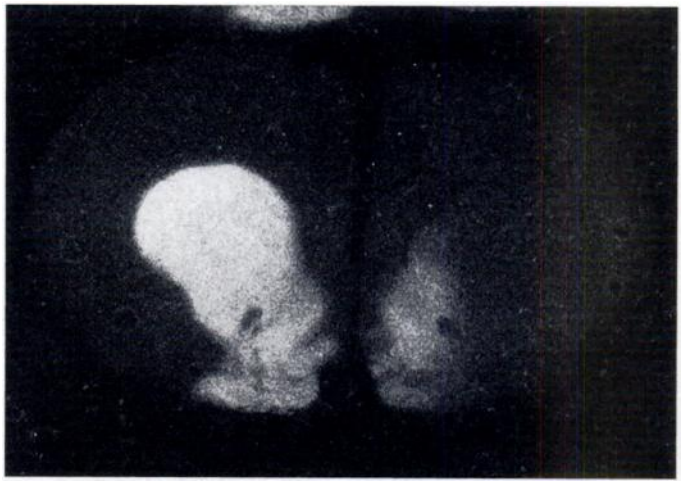

Fig. 3. *In vitro* autoradiographic analysis of the striatal [125I]RTI-121 binding in untreated (*left*) and 6-OHDA-treated (*right*) rat striatum. The autoradiograph was generated by incubation of mounted rat brain slices with 15 pM [125I]RTI-121, as described in Materials and Methods. Intense labeling of striatal DATs is present in the untreated brain, whereas little or no label can be observed in the 6-OHDA-treated striatum.

(four experiments), using a fixed concentration of labeled RTI-121 (15 pM) and increasing concentrations of unlabeled RTI-121 (1 pM to 100 nM). The two-component model of the data was statistically preferred over a single-component model ($p < 0.01$, F test). Scatchard transformation of the data indicated that the affinities in mouse tissue were similar to those observed in rat tissue. The affinity of the high affinity, low capacity component was 0.12 ± 0.02 pM (mean ± standard error), and the affinity for the low affinity, high capacity component was 2.48 ± 0.75 nM. The numbers of high and low affinity mouse striatal sites labeled by [^{125}I]RTI-121 were 128 ± 33 and 637 ± 168 pmol/g of tissue (original wet weight), respectively.

Pharmacological analysis of specific [^{125}I]RTI-121 binding. The pharmacological profile of specific [^{125}I]RTI-121 binding in the rat striatum was studied using a wide variety of drugs that are known to bind to the DAT, NE transporter, and 5-HT transporter. The overall pattern of inhibition of specific [^{125}I]RTI-121 binding was characteristic for the DAT (Table 1). Specifically bound [^{125}I]RTI-121 was displaced by (−)-cocaine with a potency that was approximately 270 times greater than the potency of the (+)-isomer. The rank order of potency for the two-site cocaine congeners was RTI-121 > WIN 35,428 > (−)-cocaine ≫ (+)-cocaine. The single-site DA uptake inhibitors displaced specific [^{125}I]RTI-121 binding with a rank order of GBR 12935 = GBR 12909 > mazindol > benztropine > nomifensine, consistent with the pharmacology of the DAT. Upon examination of total [^{125}I]RTI-121 binding (Fig. 4), it was noted that cocaine and its two-site analogs displaced approximately 95% of the bound ligand, whereas GBR 12909, nomifensine, and benztropine displaced total [^{125}I]RTI-121 binding by 82%, 86%, and 88%, respectively. Unexpectedly, mazindol and GBR 12935 displaced [^{125}I]RTI-121 binding by 95%.

TABLE 1

Inhibition by various monoamine uptake inhibitors of specific [^{125}I]RTI-121 binding

The inhibition of 15 pM [^{125}I]RTI-121 binding to 0.1 mg of striatal membranes by various competing compounds was determined as described in Materials and Methods. The IC_{50} and Hill slope values were determined using the EBDA computer program. Values represent the mean of three to five experiments, each performed in triplicate.

	IC_{50}	Hill slope
	nM	
DA uptake inhibitors		
Compounds with two binding sites		
1 (−)-Cocaine	82.65 ± 1.23	0.82 ± 0.08
2 (+)-Cocaine	21,900 ± 427	0.78 ± 0.10
3 WIN 35,428	11.6 ± 0.13	0.82 ± 0.02
4 RTI-121	0.28 ± 0.02	0.84 ± 0.02
5 Benztropine	27.8 ± 2.97	0.78 ± 0.02
Compounds with one binding site		
6 Nomifensine	37.5 ± 1.47	0.97 ± 0.06
7 Mazindol	5.40 ± 0.56	0.93 ± 0.01
8 GBR 12909	0.31 ± 0.02	0.74 ± 0.03
9 GBR 12935	0.11 ± 0.01	0.85 ± 0.04
NE uptake inhibitors		
10 Talsupram	4,410 ± 145	1.04 ± 0.07
11 Desipramine	2,190 ± 75.4	1.02 ± 0.02
12 Nisoxetine	214 ± 15.3	1.03 ± 0.09
13 Tomoxetine	243 ± 37.4	0.67 ± 0.04
5-HT uptake inhibitors		
14 Citalopram	6,470 ± 175	1.41 ± 0.11
15 Imipramine	4,320 ± 194	0.91 ± 0.03
16 Amitriptyline	1,560 ± 67.8	1.02 ± 0.09
17 Nortriptyline	1,120 ± 84.0	0.94 ± 0.06
18 Chloripramine	767 ± 7.51	1.00 ± 0.02
19 Paroxetine	184 ± 21.6	1.01 ± 0.11

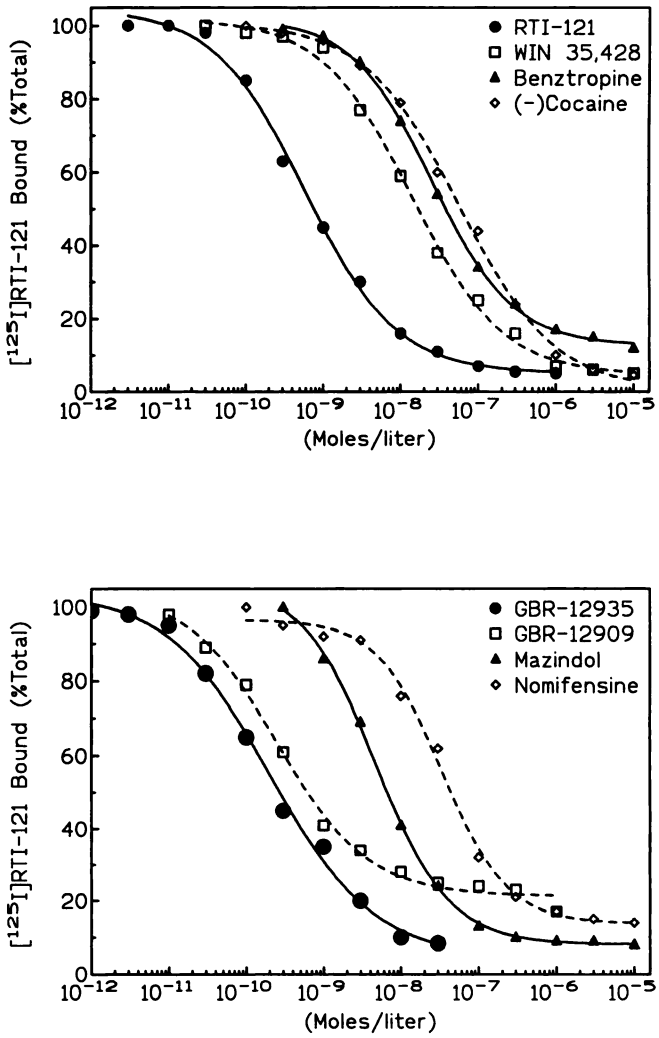

Fig. 4. Displacement of RTI-121 binding in rat striatum by various DA uptake inhibitors. Striatal membranes were incubated with 15 pM [^{125}I]RTI-121 and increasing amounts of various inhibitors. The results presented are the mean of three independent experiments, each determined in triplicate. The fit of this plot was determined using EBDA. *Open symbols*, cocaine and its analogs; *closed symbols*, non-cocaine-like compounds.

Drugs that act upon the NE and 5-HT transporters were not potent displacers of specific [^{125}I]RTI-121 binding in the striatum. Further analysis revealed a highly significant correlation ($r = 0.98$, $p < 0.0001$) between the ability of drugs to displace specific [^{125}I]RTI-121 binding and their potencies as DA reuptake blockers (Fig. 5) (20, 36, 37). No such correlation could be obtained with the potencies to inhibit either NE reuptake ($r = -0.12$, $p = 0.66$) or 5-HT reuptake ($r = -0.09$, $p = 0.75$).

Distribution of specific [^{125}I]RTI-121 binding in rat brain. The distribution of [^{125}I]RTI-121 binding in the rat brain closely paralleled the distribution of DA nerve terminals in the rat brain (Fig. 6). Areas such as the striatum, nucleus accumbens, olfactory tubercle, and substantia nigra, which are all rich in dopaminergic nerve terminals, were labeled. The degree of labeling was proportional to the num-

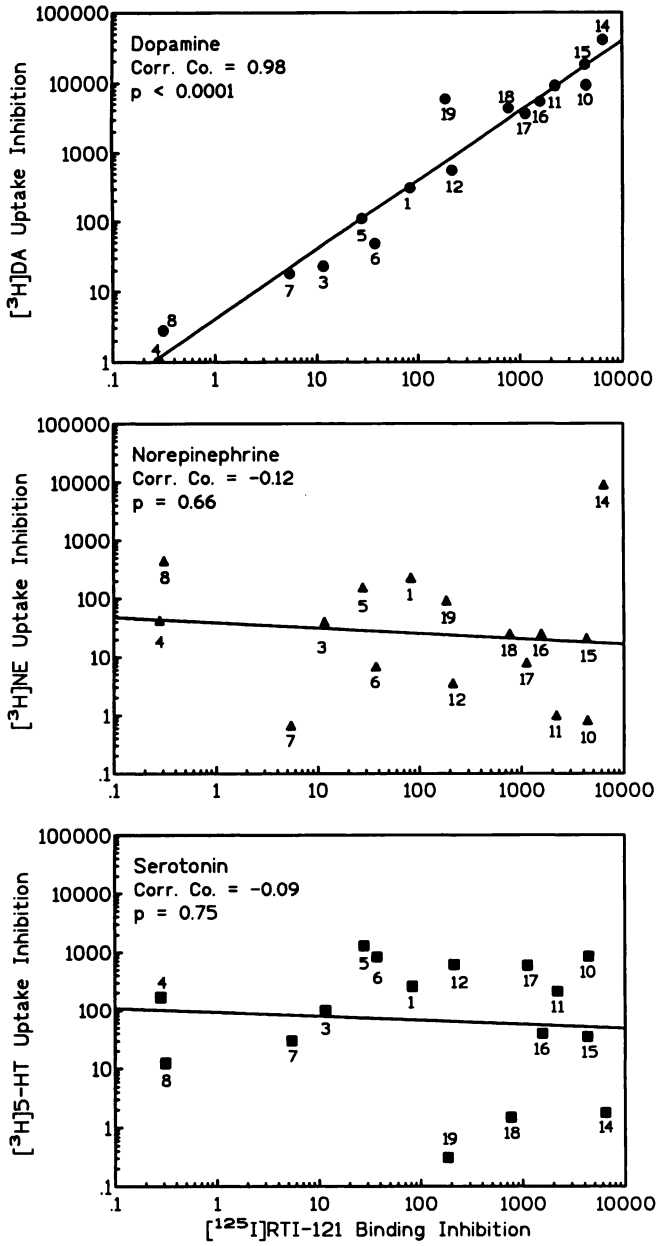

Fig. 5. Correlation of the potencies to displace striatal [125I]RTI-121 binding and inhibit the uptake of either DA (*top*), NE (*middle*), or 5-HT (*bottom*). **1**, (−)-Cocaine; **3**, WIN 35,428; **4**, RTI-121; **5**, benztropine; **6**, nomifensine; **7**, mazindol; **8**, GBR 12909; **10**, talsupram; **11**, desipramine; **12**, nisoxetine; **14**, citalopram; **15**, imipramine; **16**, amitriptyline; **17**, nortriptyline; **18**, chloripramine; **19**, paroxetine. Values for inhibition are from reports by Anderson (20) and Hytell and Larsen (36, 37) and Boja *et al.* (31).

ber of dopaminergic neurons in that area. The rank order of labeling was striatum > nucleus accumbens ≫ olfactory tubercle > substantia nigra. Areas without dopaminergic neurons were not labeled.

This pattern of distribution is in sharp contrast to the brain areas labeled by [125I]RTI-55 (compare Fig. 5, *top* and *bottom*). Whereas dopaminergic neuron-rich areas were labeled by [125I]RTI-55, areas that are rich in serotonergic neurons, such as the cerebral cortex, hippocampus, brainstem, hypothalamus, and thalamus, were also intensely labeled. The areas most intensely labeled by [125I]RTI-55 include the substantia nigra, superior colliculus, striatum, and brainstem. The entire midbrain area, cerebral cortex, and hippocampus were also labeled.

Discussion

We previously developed [125I]RTI-55 as a high affinity iodinated ligand for the DAT (10, 11). However, its affinity for the 5-HT (11, 26–29) and NE (29) transporters limited its usefulness as a selective DAT probe. As part of our ongoing characterization of the cocaine binding sites located on the DAT, we undertook to develop a new ligand that would be superior to RTI-55 in its selectivity for the DAT. The results of this study demonstrate the usefulness of [125I]RTI-121 in this regard. Thus, like cocaine (4–7), WIN 35,428 (8), WIN 35,065–2 (9), and RTI-55 (10, 11), which reveal high and low affinity binding components and biphasic association/dissociation kinetics, [125I]RTI-121 associates and dissociates from the DAT in two phases and binds to both high and low affinity components.

In the present experiments, with a concentration of 15 pM [125I]RTI-121 approximately 10% of the high affinity sites are labeled, whereas only 1% of the low affinity sites are labeled; this means that [125I]RTI-121 is bound to approximately 29 pmol/g of the high affinity sites and approximately 4.4 pmol/g of the low affinity sites. At the present time it is not known whether there is a difference in pharmacology between the high and low affinity sites. However, when either the high or low affinity values of labeled compounds were compared with the IC_{50} of the unlabeled compound to displace [3H]WIN 35,428 from the DAT, there was a strong correlation for both the high and low affinity values (r = 0.95 and 0.98, respectively).[4] The stronger correlation of the low affinity value may be due to the higher absolute number of low affinity sites.

Previous work has determined that the two binding sites observed for the cocaine analog [3H]WIN 35,428 arise from a single expressed cDNA (38). Calligaro and co-workers (6, 39) suggested that the cocaine binding site is an allosteric protein, with DA and Na^+ acting as allosteric modulators that stabilize the lower affinity state for cocaine. There is some evidence to support the idea that the high and low affinity binding states for cocaine and its analogs actually represent two interchangeable affinity states, rather than two distinct sites. Pristupa *et al.* (24) compared K_i values of various compounds for the inhibition of [3H]DA uptake with the K_i values for the inhibition of [3H]WIN 35,428 binding; a strong correlation was found between the K_i values of the various uptake blockers for inhibition of the high affinity component of binding and the K_i values for inhibition of [3H]DA uptake. In an elegant series of experiments by Laruelle *et al.* (40), the effect of temperature upon the ratio of high affinity to low affinity states was demonstrated. The number of high affinity [125I]RTI-55 binding sites in baboon striatum was reduced when the incubation temperature was raised from 22° to 37°. The reduction in the number of high affinity sites was accompanied by an increase in the number of low affinity [125I]RTI-55 binding sites. An additional experiment demonstrated partial reversal of the temperature effect observed, in that tissue preincubated at 37° and then assayed at 22°

[4] J. W. Boja, unpublished observations.

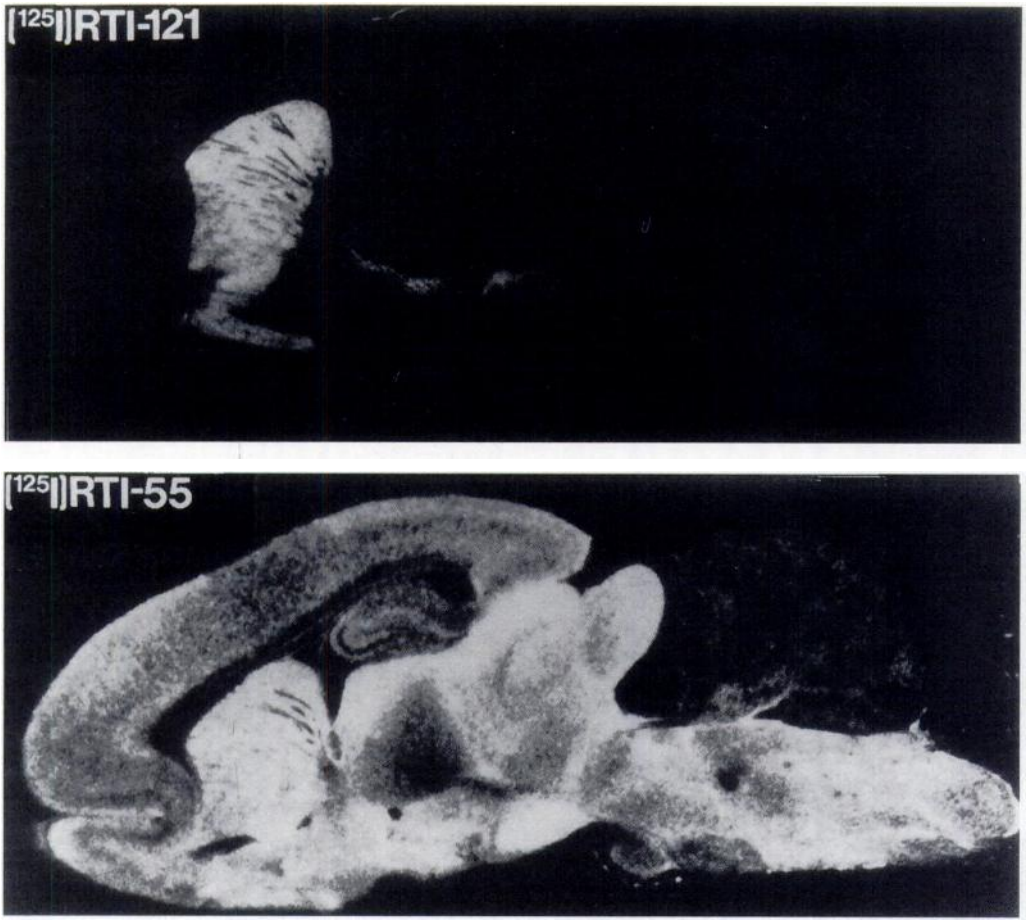

Fig. 6. *In vitro* autoradiographic distribution of [^{125}I]RTI-121 (*top*) and [^{125}I]RTI-55 (*bottom*) binding. The autoradiographs were generated by incubation of mounted rat brain slices with 15 pM concentrations of either [^{125}I]RTI-121 or [^{125}I]RTI-55, as described in Materials and Methods. The distribution of [^{125}I]RTI-121 was limited to areas rich in dopaminergic innervation, such as the striatum, nucleus accumbens, olfactory tubercle, and substantia nigra. With [^{125}I]RTI-55, dopaminergic neuron-rich areas were labeled and areas that are rich in serotonergic neurons, such as the cerebral cortex, hippocampus, brainstem, hypothalamus, and thalamus, were also intensely labeled. The areas of most intense labeling include the substantia nigra, superior colliculus, striatum, and brainstem. Weaker labeling was observed in the entire midbrain area, cerebral cortex, and hippocampus.

revealed a partial (72%) return to control values. Separation of the high affinity and low affinity components of [^{3}H]WIN 35,428 binding using an isothiocyanate analog of cocaine has been reported (41). Preincubation of rat striatal membranes with 1 μM *m*-isothiocyanatobenzylecgonine methyl ester reduced but did not eliminate the high affinity component of [^{3}H]WIN 35,428 binding. In addition, increasing the concentration of *m*-isothiocyanatobenzylecgonine methyl ester to either 10 or 100 μM completely eliminated the high affinity component.

It has been suggested that cocaine binds to a site different from those recognized by either GBR-12935 or mazindol (17, 18, 20–22, 24, 25) and that these sites may be overlapping allosteric sites. In contrast, Reith and co-workers (42, 43) suggested that there is only a single site that cocaine, mazindol, and GBR-12935 recognize. Furthermore, the single site recognized by these compounds exists only in the high affinity state. However, the concept of a single high affinity binding component for cocaine is at odds with the present data and those of others (4–8, 10, 11, 15, 24, 28). More likely, the cocaine binding site exists in both high and low affinity states and certain conditions favor the observation of either one or two affinity states.

The observed difference in the ratio of high to low affinity sites between rats and mice may simply be a species difference. Because identical binding conditions were used in both experiments, it seems likely that the difference in binding ratios observed is an effect other than a species difference. Recently, Reith and Selmeci (42) suggested that the binding properties of the high affinity component may indeed be species specific and that a species difference in the DAT may also account for the lack of complete inhibition of [^{3}H]GBR 12395 binding in monkeys, compared with mice. Indeed, there seems to be 93% homology between the human and rat DATs at the amino acid level (44). If a similar or greater difference exists between the monkey and mouse DATs, then the binding properties of the two transporters would be expected to differ.

[^{125}I]RTI-121 labels a similar number of sites as does [^{125}I]RTI-55 (11) in rat striatum and slightly more sites than does [^{3}H]WIN 35,428 (8); however, this difference is most likely due to the use of Tris buffer in the [^{3}H]WIN 35,428 study, as well as a different species (monkey). Tris buffer is known to affect the binding capacity of a number of ligands (39, 45), including cocaine.

The pharmacological profile of [^{125}I]RTI-121 binding in the

striatum is similar to that of [3H]cocaine (4–7), [3H]WIN 35,428 (8), [3H]WIN 35,065–2 (9), and [3H]RTI-55 (10, 11). Specifically, compounds known to inhibit DA reuptake are effective inhibitors of [125I]RTI-121 binding, with roughly the same rank order as their potencies to inhibit [3H]cocaine binding at the DAT (Table 1, **1-9**), whereas compounds classified as either NE or 5-HT reuptake inhibitors are substantially less potent in inhibiting [125I]RTI-121 binding (Table 1, **10-13** and **14-19**, respectively). Moreover, the functional significance of RTI-121 as a selective inhibitor of DA reuptake is confirmed by the robust correlation ($r = 0.98$) of the radioligand binding data for seven DA reuptake inhibitors, three NE reuptake inhibitors, and six 5-HT reuptake inhibitors with their potencies to inhibit DA reuptake. No correlation was apparent for either the NE or 5-HT transporter. These observations, taken together, indicate that [125I]RTI-121 binds to the DAT.

The autoradiographic distribution of [125I]RTI-121 binding in the rat brain is also consistent with that of the DAT. [125I]RTI-121 binding is observed in areas of the brain rich in dopaminergic neurons, and it is absent in other brain areas. Comparison of [125I]RTI-121 and [125I]RTI-55 binding under identical autoradiographic conditions confirms the greater selectivity of [125I]RTI-121 for the DAT. The pattern of distribution to the dopaminergic system seen within rat brain was also observed in autoradiographic images of human brain obtained using [125I]RTI-121.[5] A similar pattern of distribution was observed using the selective cocaine analog [3H]WIN 35,428 (46). In contrast, imaging studies using [125I]RTI-55 revealed the distribution of both DATs and 5-HT transporters (11, 27). Thus, because [125I]RTI-55 has a distribution pattern resembling that of cocaine, it is useful for examining the overall distribution of cocaine binding sites, whereas the high affinity, low background binding, and superior selectivity of [125I]RTI-121 make it an excellent ligand for the study of the DAT.

The present study also demonstrates that, with the exception of mazindol and GBR 12935, noncocaine ligands do not fully displace [125I]RTI-121. These findings are consistent with, and expand upon, a previous report (8). Specifically, whereas cocaine and structurally similar analogs displace 95% of total [125I]RTI-121 binding, noncocaine analogs such as GBR 12909, nomifensine, and benztropine displace [125I]RTI-121 by only 82%, 86%, and 88%, respectively. Thus, it appears that these agents do not recognize all sites labeled by [125I]RTI-121, in agreement with the suggestion (8) that noncocaine ligands may recognize only a subset of sites labeled by cocaine congeners. Cocaine itself appears to recognize sites other than those labeled by mazindol (23) and GBR 12935 (24, 25). Additional work is needed to fully understand the implications of these findings.

The results of this study indicate that [125I]RTI-121 may be a preferred ligand for the DAT. Its very high affinity, low background binding, and superior selectivity for the DAT make it an excellent ligand for the study of the DAT.

[5] J. Staley, J. W. Boja, F. I. Carroll, H. Seltzman, C. D. Wyrick, A. H. Lewin, P. Abraham, and D. C. Mash. Mapping the dopamine transporter in human brain with the novel cocaine analog [125I]RTI-121, manuscript in preparation.

Acknowledgments

The authors would like to thank Drs. Bertha Madras and Maarten Reith for generously supplying a sample of talsupram and Drs. Beverly Brown and Garth Brown of NEN for supplying [125I]RTI-121 and [125I]RTI-55.

References

1. Reith, M. E. A., B. E. Meisler, H. Sershen, and A. Lajtha. Saturable [3H]cocaine binding in central nervous system of mouse. *Life Sci.* **27**:1055–1062 (1980).
2. Kennedy, L. T., and I. Hanbauer. Sodium sensitive cocaine binding to rat striatal membrane: possible relationship to dopamine uptake sites. *J. Neurochem.* **41**:172–178 (1983).
3. Reith, M. E. A., B. E. Meisler, H. Shershen, and A. Lajtha. Structural requirements for cocaine congeners to interact with dopamine and serotonin uptake sites in mouse brain and to induce stereotyped behavior. *Biochem. Pharmacol.* **35**:1123–1129 (1986).
4. Schoemaker, H., C. Pimoule, S. Arbilla, B. Scatton, F. Javoy-Agid, and S. Z. Langer. Sodium dependent [3H]cocaine binding associated with dopamine uptake sites in the rat striatum and human putamen decreases after dopaminergic denervation and in Parkinson's disease. *Naunyn Schmiedebergs Arch. Pharmacol.* **329**:227–235 (1985).
5. Calligaro, D. O., and M. E. Eldefrawi. Central and peripheral cocaine receptors. *J. Pharmacol. Exp. Ther.* **243**:61–68 (1987).
6. Calligaro, D. O., and M. E. Eldefrawi. High affinity stereospecific binding of [3H]cocaine in striatum and its relationship to the dopamine transporter. *Membr. Biochem.* **7**:87–106 (1988).
7. Madras, B. K., M. A. Fahey, J. Bergman, D. R. Canfield, and R. D. Spealman. Effects of cocaine and related drugs in nonhuman primates. I. [3H]Cocaine binding sites in caudate-putamen. *J. Pharmacol. Exp. Ther.* **251**:131–141 (1989).
8. Madras, B. K., R. D. Spealman, M. A. Fahey, J. L. Neumeyer, J. K. Saha, and R. A. Milius. Cocaine receptors labeled by [3H]2β-carbomethoxy-3β-(4-fluorophenyl)tropane. *Mol. Pharmacol.* **36**:518–524 (1989).
9. Ritz, M. C., R. J. Lamb, S. R. Goldberg, and M. J. Kuhar. Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science (Washington D. C.)* **237**:1219–1223 (1987).
10. Boja, J. W., A. Patel, F. I. Carroll, M. A. Rahman, A. Philip, A. H. Lewin, and M. J. Kuhar. [125I]RTI-55: a potent ligand for dopamine transporters. *Eur. J. Pharmacol.* **194**:133–134 (1991).
11. Boja, J. W., W. M. Mitchell, A. Patel, T. A. Kopajtic, F. I. Carroll, A. H. Lewin, P. Abraham, A. H. Lewin, and M. J. Kuhar. High affinity binding of [125I]RTI-55 to dopamine and serotonin transporters in rat brain. *Synapse* **12**:27–36 (1992).
12. Carroll, F. I., A. H. Lewin, J. W. Boja, and M. J. Kuhar. Cocaine receptor: biochemical characterization and structure-activity relationships of cocaine analogs at the dopamine transporter. *J. Med. Chem.* **35**:969–981 (1992).
13. Misra, A. L., V. V. Giri, M. N. Patel, V. R. Alluri, and S. J. Mule. Disposition and metabolism of [3H]cocaine in acutely and chronically treated monkeys. *Drug Alcohol Depend.* **2**:261–272 (1977).
14. Fischman, M. W., C. R. Schuster, L. Resnekov, J. F. E. Shick, N. A. Krasnegor, W. Fennel, and D. X. Freedman. Cardiovascular and subjective effects of intravenous cocaine administration in humans. *Arch. Gen. Psychiatry* **33**:983–989 (1976).
15. Ritz, M. C., J. W. Boja, D. Grigoriadis, F. I. Carroll, A. H. Lewin, and M. J. Kuhar. [3H]WIN 35,065–2: a ligand for cocaine receptors in striatum. *J. Neurochem.* **55**:1556–1562 (1990).
16. Bergman, J., B. K. Madras, S. E. Johnson, and R. D. Spealman. Effects of cocaine and related drugs in nonhuman primates. III. Self-administration by squirrel monkeys. *J. Pharmacol. Exp. Ther.* **251**:150–155 (1989).
17. Javitch, J. A., R. O. Blaustein, and S. H. Snyder. [3H]Mazindol binding associated with neuronal dopamine and norepinephrine uptake sites. *Mol. Pharmacol.* **26**:35–44 (1984).
18. Dubocovich, M. L., and N. R. Zahniser. Binding characteristics of the dopamine uptake inhibitor [3H]nomifensine to striatal membranes. *Biochem. Pharmacol.* **34**:1137–1144 (1985).
19. Scherwi, M. M., P. Skolnick, M. F. Rafferty, K. C. Rice, A. J. Janowsky, and S. M. Paul. [3H]*Threo*-(±)-methylphenidate binding to 3,4-dihydroxyphenylethylamine uptake sites in corpus striatum: correlation with the stimulant properties of ritalinic acid esters. *J. Neurochem.* **45**:1062–1070 (1985).
20. Anderson, P. H. Biochemical and pharmacological characterization of [3H]GBR-12935 binding *in vitro* to rat striatal membrane: labeling of the dopamine uptake complex. *J. Neurochem.* **48**:1887–1896 (1987).
21. Anderson, P. H. The uptake inhibitor [3H]GBR-12935: selectivity and molecular mechanism of action. *Eur. J. Pharmacol.* **166**:493–504 (1989).
22. Niznik, H. B., R. F. Tyndale, F. R. Sallee, F. J. Gonzales, J. P. Hardwick, T. Inaba, and W. Kalow. The dopamine transporter and cytochrome P450IIDI (debrisoquine-4-hydroxylase) in brain: resolution and identification of two distinct [3H]GBR-12935 binding proteins. *Arch. Biochem. Biophys.* **276**:424–432 (1990).

23. Wilson, J. M., J. N. Norrega, M. E. Carroll, H. B. Niznik, K. Shannak, S. T. Lac, Z. B. Pristupa, L. M. Dixon, and S. J. Kish. Heterogeneous subregional binding patterns of ^{3}H-WIN 35,428 and ^{3}H-GBR 12,935 are differently regulated by chronic cocaine self- administration. *J. Neurosci.* **14:** 2966–2979 (1994).
24. Pristupa, Z. B., J. M. Wilson, B. J. Hoffman, S. J. Kish, and H. B. Niznik. Pharmacological heterogeneity of the cloned and native human dopamine transporter: disassociation of [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 binding. *Mol. Pharmacol.* **45:**125–135 (1994).
25. Allard, P., M. Danielsson, K. Papworth, and J. O. Marcusson. [^{3}H]GBR-12935 binding to cerebral cortex is not to dopamine uptake sites. *J. Neurochem.* **62:**338–341 (1994).
26. Scheffel, U., R. F. Dannals, E. J. Cline, G. A. Ricaurte, F. I. Carroll, P. Abraham, A. H. Lewin, and M. J. Kuhar. [$^{123/125}$I]RTI-55, an *in vivo* label for the serotonin transporter. *Synapse* **11:**134–139 (1991).
27. Cline, E. J., U. Scheffel, J. W. Boja, W. M. Mitchell, F. I. Carroll, P. Abraham, A. H. Lewin, and M. J. Kuhar. *In vivo* binding of [^{125}I]RTI-55 to dopamine transporters: pharmacology and regional distribution with autoradiography. *Synapse* **12:**37–46 (1992).
28. Wall, S. C., R. B. Innis, and G. Rudnik. Binding of the cocaine analog 2β-carbomethoxy-3β-(4-[^{125}I]iodophenyl)tropane to serotonin and dopamine transporters: differentionic requirements for substrate and 2β-carbomethoxy-3β-(4-[^{125}I]iodophenyl) tropane binding. *Mol. Pharmacol.* **43:**264–270 (1992).
29. Gu, H., S. C. Wall, and G. Rudnick. Stable expression of biogenic amine transporters reveals differences in inhibitor sensitivity, kinetics and ion dependence. *J. Biol. Chem.* **269:**7124–7130 (1994).
30. Carroll, F. I., P. Abraham, A. H. Lewin, K. A. Parham, J. W. Boja, and M. J. Kuhar. Isopropyl and phenyl esters of 3β-(4-substituted phenyl)tropan-2β-carboxylic acids: potent and selective compounds for the dopamine transporter. *J. Med. Chem.* **35:**2497–2500 (1992).
31. Boja, J. W., R. M. McNeil, A. H. Lewin, P. Abraham, F. I. Carroll, and M. J. Kuhar. Selective dopamine transporter inhibition by cocaine analogs. *NeuroReport* **3:**984–986 (1992).
32. Scheffel, U., R. F. Dannals, D. F. Wong, F. Yokoi, F. I. Carroll, and M. J. Kuhar. Dopamine transporter imaging with novel, selective cocaine analogs. *NeuroReport* **3:**969–972 (1992).
33. Cadet, J. L., and S. M. Zhu. The intrastriatal 6-hydroxydopamine model of hemiparkinsonism: quantitative receptor autoradiographic evidence of correlation between circling behavior and presynaptic as well as postsynaptic nigrostriatal markers in the rat. *Brain Res.* **595:**316–326 (1992).
34. Kuhar, M. J., and J. R. Unherstall. Receptor autoradiography, in *Methods in Neurotransmitter Analysis* (H. Yamamura, ed.). Raven Press, New York, 177–218 (1990).
35. Carroll, F. I., M. A. Rahman, P. Abraham, K. Parham, A. H. Lewin, R. F. Dannals, E. Shaya, U. Scheffel, D. F. Wong, J. W. Boja, and M. J. Kuhar. [^{123}I]3β-(4-Iodophenyl)tropan-2β-carboxylic acid methyl ester (RTI-55), a unique cocaine receptor ligand for imaging the dopamine and serotonin transporters *in vivo*. *Med. Chem. Res.* **1:**289–294 (1991).
36. Hytell, J. Citalopram: pharmacological profile of a specific serotonin uptake inhibitor with antidepressant activity. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **6:**277–295 (1982).
37. Hytell, J., and J. J. Larsen. Neurochemical profile of Lu 19–005, a potent inhibitor of uptake of dopamine, noradrenaline, and serotonin. *J. Neurochem.* **44:**1615–1622 (1985).
38. Boja, J. W., L. Markham, A. Patel, G. Uhl, and M. J. Kuhar. Expression of a single dopamine cDNA can confer two cocaine binding sites. *NeuroReport* **3:**247–248 (1992).
39. Eshleman, A. J., D. O. Calligaro, and M. E. Eldefrawi. Allosteric regulation by sodium of the binding of [^{3}H]cocaine and [^{3}H]GBR 12935 to rat and bovine striata. *Membr. Biochem.* **10:**129–144 (1993).
40. Laruelle, M., S. S. Giddings, Y. Zoa-Ponce, D. S. Charney, J. L. Neumeyer, R. M. Baldwin, and R. B. Innis. Methyl-3β-(4-[^{125}I]iodophenyl)tropan-2β-carboxylate *in vitro* binding to dopamine and serotonin transporters under physiological conditions. *J. Neurochem.* **62:**978–986 (1994).
41. Boja, J. W., M. A. Rahman, P. Abraham, A. H. Lewin, F. I. Carroll, and M. J. Kuhar. Isothiocynate derivatives of cocaine: irreversible inhibition of ligand binding at the dopamine transporter. *Mol. Pharmacol.* **39:**339–345 (1991).
42. Reith, M. E. A., and G. Selmeci. Radiolabeling of dopamine uptake sites in mouse striatum: comparison of binding sites for cocaine, mazindol, and GBR 12935. *Naunyn Schmiedebergs Arch. Pharmacol.* **345:**309–318 (1992).
43. Reith, M. E. A., B. de Costa, K. C. Rice, and A. E. Jacobson. Evidence for mutually exclusive binding of cocaine, BTCP, GBR 12935 and dopamine to the dopamine transporter. *Eur. J. Pharmacol.* **277:**417–425 (1992).
44. Giros, B., and M. G. Caron. Molecular characterization of the dopamine transporter. *Trends Pharmacol. Sci.* **14:**43–49 (1993).
45. Reith, M. E. A., B. E. Meisler, H. Sershen, and A. Lajtha. [^{3}H]Cocaine binding in brain is inhibited by tris(hydroxymethyl)aminomethane. *J. Neurosci. Methods* **12:**151–153 (1984).
46. Canfield, D. R., R. D. Spealman, M. J. Kaufman, and B. K. Madras. Autoradiographic localization of cocaine binding sites by [^{3}H]CFT ([^{3}H]WIN 35,428) in the monkey brain. *Synapse* **6:**189–195 (1990).

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