[^{3}H]WIN 35,065-2: A Ligand for Cocaine Receptors in Striatum

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Abstract: [^{3}H]WIN 35,065-2 binding to striatal membranes was characterized, primarily by centrifugation assay. Like [^{3}H]cocaine, [^{3}H]WIN 35,065-2 binds to both high- and low-affinity sites. [^{3}H]WIN 35,065-2, however, exhibits consistently higher affinities than [^{3}H]cocaine. Saturation experiments indicate a low-affinity binding site with an apparent K_D of ~160 nM and a B_{max} of 135 fmol/mg of tissue. A high-affinity site has also been identified with an apparent K_D of 5.6 nM and a B_{max} of 5.2 fmol/mg of tissue. The specific-to-nonspecific binding ratios with [^{3}H]WIN 35,065-2 were higher than with [^{3}H]cocaine in both centrifugation and filtration assays. Pharmacological characterization suggests that [^{3}H]WIN 35,065-2 binds to the dopamine transporter. Mazindol, GBR 12909, nomifensine, and (−)-cocaine are potent inhibitors of [^{3}H]WIN 35,065-2 binding. In contrast, the norepinephrine transporter ligand desipramine is a weak inhibitor, and the serotonin transporter ligand citalopram does not inhibit binding. The effect of sodium on binding was examined under conditions in which (a) the low-affinity site was primarily (87%) occupied and (b) ~50% of both sites were occupied. The results indicate that both sites are sodium dependent. Injection of 6-hydroxydopamine into the striatum results in a significant loss of both high- and low-affinity sites, a finding suggesting that both sites are on dopaminergic nerve terminals. Taken together, these data are consistent with the presence of multiple cocaine binding sites associated with the dopamine transporter. **Key Words:** [^{3}H]WIN 35,065-2—Cocaine—Dopamine transporter—Cocaine receptors. **Ritz M. C. et al.** [^{3}H]WIN 35,065-2: A ligand for cocaine receptors in striatum. *J. Neurochem.* **55,** 1556–1562 (1990).

Several cocaine binding sites have been identified in both brain and periphery. In particular, [^{3}H]cocaine binding sites in striatum with micromolar affinities have been described (Kennedy and Hanbauer, 1983; Sershen et al., 1984; Shoemaker et al., 1985). These studies, using various lesions as well as correlations between inhibition of [^{3}H]cocaine binding and inhibition of [^{3}H]dopamine uptake, have shown that the binding sites are primarily associated with the dopamine uptake mechanism. We have shown previously that the cocaine binding site on the dopamine transporter appears to be associated with the reinforcing and potentially addicting properties of cocaine (Ritz et al., 1987).

More recently, radiolabeled cocaine has been used as a ligand to label its binding sites in vivo. Positron emission tomography scans using [^{11}C]cocaine revealed high concentrations of binding sites in the caudate nucleus (Fowler et al., 1989). The binding has characteristics of binding to the dopamine transporter as expected. However, the cocaine analog [^{3}H]WIN 35,065-2 has been shown to exhibit much higher specific binding than cocaine in in vivo binding experiments (Scheffel et al., 1989). This result would be expected, because WIN 35,065-2 and WIN 35,981, the phenyltropane analogs of cocaine and norcocaine, respectively, exhibit higher potencies than cocaine for inhibiting both [^{3}H]mazindol and [^{3}H]GBR 12935, ligands commonly used to label the dopamine transporter (Ritz et al., 1987; Sharkey et al., 1987; Ritz and Kuhar, 1989). In addition, WIN 35,065-2 and WIN 35,428 also inhibit brain [^{3}H]cocaine binding (Sershen et al., 1982), and [^{3}H]WIN 35,428 has been shown to label binding sites associated with the dopamine transporter (Madras et al., 1989a). Finally, the ester linkage of the benzoyl function of cocaine has been eliminated in these analogs by direct attachment of the phenyl group to the tropane ring, making them more resistant to metabolic and chemical degradation (Fig. 1). In this study, we determined the binding characteristics of
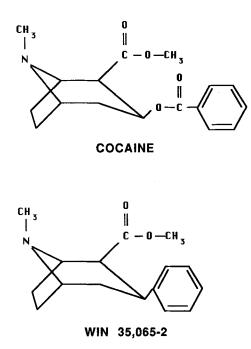
COCAINE

WIN 35,065-2

FIG. 1. Structures of cocaine and WIN 35,065-2.

[³H]WIN 35,065-2 to striatal membranes in vitro, using a centrifugation assay.

[³H]Cocaine has both high- and low-affinity sites in striatal tissue (Calligaro and Eldefrawi, 1987, 1988), and pharmacological data suggest that both sites are associated with the dopamine transporter. [³H]WIN 35,065-2 is an important ligand to use for studying these sites, because it does not have a higher affinity at the serotonin or norepinephrine transporter, relative to the dopamine transporter, as do [³H]cocaine and some other analogs such as WIN 35,428 and WIN 35,981 (Ritz et al., 1987). [³H]Cocaine binding sites that appear to be associated with the serotonin transporter have been identified in cortical tissues by other investigators (Reith et al., 1980, 1983, 1984, 1985). Specifically, these reports suggest that lesions of serotonergic neurons may decrease the number of these cocaine binding sites in mouse cortex. Significant correlations between inhibition of [³H]cocaine binding in cortex and inhibition of serotonin uptake are also discussed. In addition, these studies suggest important similarities between the binding of [³H]cocaine and the serotonin transporter ligand [³H]imipramine. Thus, another goal of this study was to obtain additional evidence that both high- and low-affinity cocaine sites are specifically associated with the dopamine transporter and dopaminergic nerve terminals.

MATERIALS AND METHODS

Materials

[³H]WIN 35,065-2 (85.0 Ci/mmol) was obtained in ethanol from New England Nuclear (Boston, MA, U.S.A.). Standard purity checks by the vendor documented that the specific activity of the product progressively decreased to 85% in 1

year. The ligand was also synthesized by methylation of 3β-phenyl-1αH,5αH-nortropane-2β-carboxylate (Clarke et al., 1973) with [³H]CH₃I. After synthesis, the identity of [³H]WIN 35,065-2 was established by its coelution on TLC and gas chromatography with an authentic sample. Analysis by TLC-radioscan and gas chromatography showed the product to be >98% pure, and it was subsequently stored in toluene. The specific activity was 25 Ci/mmol. Experimental details of the synthesis will be described in a subsequent publication. (+)-Cocaine, (+)-pseudococaine, and (−)-pseudococaine were prepared by previously reported procedures (Lewin et al., 1987). (−)-Cocaine and norcocaine were obtained from the National Institute on Drug Abuse. Citalopram was obtained from Lundbeck A/S (Copenhagen, Denmark), GBR 12909 from Research Biochemicals, desipramine HCl from Sigma, nomifensine from Hoechst, and mazindol from Sandoz.

Tissue preparation

Tissues for all binding experiments were dissected from the brains of male Sprague–Dawley rats, 60–120 days old. The rats were killed by decapitation, and their brains were removed and washed in cold saline. Brain regions were then dissected, frozen, and stored at −70°C until used in the assay procedures. Tissues were homogenized using a Brinkmann Polytron (Brinkmann Instruments, Westbury, NY, U.S.A.) in 20 volumes of the assay buffer and then centrifuged at 50,000 g for 10 min. The resulting pellet was resuspended in 20 volumes of the assay buffer, recentrifuged, and resuspended in buffer to yield the desired tissue concentration for addition to the assay.

[³H]WIN 35,065-2 binding

In our standard assay, homogenized tissues (∼1.5 mg original wet weight) were incubated for 1 h at 0°C in buffer (50 mM Tris, 120 mM NaCl, 5 mM KCl, pH 7.8) containing [³H]WIN 35,065-2. These conditions were adapted from those commonly used for ligand binding to dopamine transporters and cocaine binding sites (Javitch et al., 1984; Dubocovich and Zahniser, 1985; Shoemaker et al., 1985; Janowsky et al., 1986; Anderson, 1987). Nonspecific binding was defined by addition of 100 μM (−)-cocaine in each experiment. Final assay volumes were 0.5 ml. Assay mixtures were prepared and incubated in 1.5-ml polypropylene conical tubes.

For saturation experiments, the specific activity of [³H]WIN 35,065-2 was diluted fivefold with unlabeled drug. The final assay concentration of WIN 35,065-2 ranged from 1 nM to 1 μM for these studies. Tissues were incubated in the presence of 50 nM [³H]WIN 35,065-2 in experiments designed to assess tissue linearity, ligand–receptor association kinetics, effects of temperature, and competitive inhibition of [³H]WIN 35,065-2 binding. To assess the effects of Na⁺ concentration on binding, tissues were incubated in 50 mM Tris at a final [³H]WIN 35,065-2 concentration of either 0.5 or 50 nM in the presence or absence of 120 mM NaCl. In the absence of NaCl, ionic strength was maintained constant by addition of equimolar concentrations of N-methyl-D-glucamine (Sigma).

At the end of the incubation period, all assay tubes were centrifuged at 12,000 g for 5 min. Supernatants were aspirated from tubes, and pellets were washed superficially without resuspension with 1 ml of cold buffer. The tip of each assay tube was cut off and placed into plastic vials, and 5 ml of scintillation fluid was added. Vials were shaken overnight, and, finally, radioactivity in each was measured by liquid scintillation spectrometry.

The data were analyzed using the nonlinear least squares curve-fitting program LIGAND (Munson and Rodbard, 1980). All analyses used single- and two-site models, with the two-site model being adopted only when it provided a statistically better fit for the experimental data than the one-site model. Mean and SE values were calculated for three to five assays.

6-Hydroxydopamine-induced striatal lesions

Male Sprague–Dawley rats (weighing 200–225 g) were anesthetized with 3 ml/kg of Chloropent (Fort Dodge Laboratories, Fort Dodge, IA, U.S.A.) and placed in a David Kopf small-animal stereotaxic apparatus. Injections of 6-hydroxydopamine (6-OHDA) were made using a 0.3-mm-diameter cannula inserted into the area of injection through a hole drilled in the skull (striatal coordinates used were as follows: anterior, 0.8 mm; lateral, 2.6 mm; ventral, 4.8 mm from the intraoccular line, sagittal suture, and dura, respectively). Injections of 2 μl over the course of 5 min were made with 6-OHDA (5 mg/ml) dissolved in buffered saline. At 1 min postinjection, the injection cannula was quickly removed. Rats were allowed to recover for 6 weeks before the striatal tissue was processed for [^{3}H]WIN 35,065-2 binding and determination of dopamine levels.

Both dopamine levels and [^{3}H]WIN 35,065-2 binding were measured in each individual animal. These were most effectively assayed simultaneously using crude P_2 tissue preparations. 6-OHDA-treated rats were killed by decapitation, and both ipsilateral and contralateral striata were dissected from brains on ice. Striata were homogenized in 20 volumes of ice-cold 0.32 *M* sucrose in a smooth glass homogenizer equipped with a motor-driven Teflon pestle. The homogenate was centrifuged at 800 *g* for 10 min at 4°C to remove large particulate material. The supernatant was decanted and centrifuged at 20,000 *g* at 4°C for 10 min, and the resultant pellet was resuspended in 50 volumes of the original wet weight in ice-cold 0.32 *M* sucrose. The homogenate was centrifuged at 50,000 *g* at 4°C for 10 min, and the resulting pellet was resuspended by sonication (Sonifier Cell Disruptor at a setting of 6 for 15 s). The homogenate was centrifuged at 50,000 *g* at 4°C for 10 min, and an aliquot (40 μl) of the supernatant was analyzed for dopamine content by the method of Zaczek and Coyle (1982). The pellet was reho-

mogenized (in a Polytron at a setting of 6 for 15 s), and 100 μl was processed for [^{3}H]WIN 35,065-2 binding as described above.
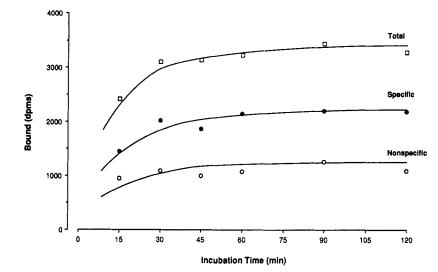
RESULTS

As tissue content in each assay tube is varied from 0.5 to 8 mg (original wet weight/tube), specific binding is proportional to tissue concentration. From a practical point of view, 1.5 mg/tube produces firm pellets after centrifugation of the assay mixture, facilitating aspiration of the supernatant without disruption of the pellet.

Figure 2 shows the results of association kinetic experiments using [^{3}H]WIN 35,065-2 concentrations of 50 n*M*. Specific binding increases rapidly and reaches equilibrium at ~30 min. At equilibrium, specific binding is ~60–65% of total binding. Our experiments also show that specific-to-nonspecific binding ratios for [^{3}H]WIN 35,065-2 are 25–30% higher than for [^{3}H]cocaine in both filtration and centrifugation assays (data not shown).

The specific binding of [^{3}H]WIN 35,065-2 to rat striatal membranes is saturable (Fig. 3). Analysis of the equilibrium binding data indicates that [^{3}H]WIN 35,065-2 binds to a heterogeneous population of binding sites in this tissue inasmuch as the data best fit a two-site model of ligand binding. When nonspecific binding is defined by addition of 100 μM cocaine, a low-affinity site exhibiting a K_D of ~160 ± 36 n*M* and a B_{max} of 135 ± 35 fmol/mg of tissue is observed. Under these conditions, a high-affinity site exhibiting an apparent K_D of ~5.6 ± 1.6 n*M* and a B_{max} of 5.2 ± 1.8 fmol/mg of tissue is also found. When nonspecific binding is defined by addition of 100 μM (−)-cocaine or 10 μM mazindol in the presence of 50 n*M* [^{3}H]WIN 35,065-2, similar amounts of specific binding are observed.

The specific binding of [^{3}H]WIN 35,065-2 to rat striatal membranes was studied at 0, 25, and 37°C.

FIG. 2. Association curve for [^{3}H]WIN 35,065-2 binding in striatum. Tissues were incubated with ligand at 50 n*M* in buffer (50 m*M* Tris, 120 m*M* NaCl, and 5 m*M* KCl) for increasing intervals. Nonspecific binding was determined by addition of 100 μM (−)-cocaine. Assays were stopped by centrifugation at the times indicated, and radioactivity associated with each condition was counted. The data presented are averages of three assays.
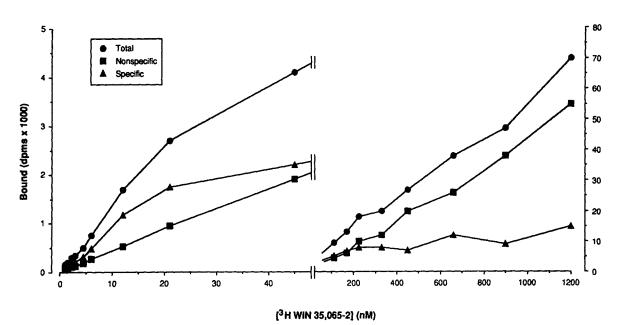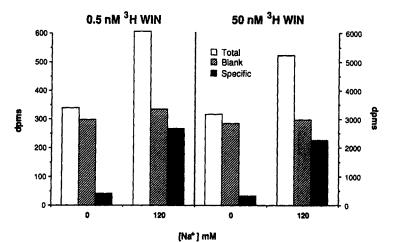
FIG. 3. Saturation curve for [³H]WIN 35,065-2 binding in striatum. Final ligand concentrations ranged from 1 nM to 1 μM. Nonspecific binding was determined by addition of 100 μM (−)-cocaine. The curve shown is representative of the data collected in saturation experiments performed as described in Materials and Methods.

The results of these experiments indicate that there were no changes in specific binding across temperature conditions, an observation indicating that the binding of [³H]WIN 35,065-2 is relatively insensitive to the effects of incubation temperature. Figure 4 indicates, however, that specific [³H]WIN 35,065-2 binding to both high- and low-affinity binding sites in rat striatal membranes is sodium dependent under these conditions. Percent receptor occupancy for each site was determined using the following formula: percent occupancy = 100 × (ligand concentration/[ligand concentration + K_D of ligand (in nM)]). After the relative abundance of high- and low-affinity sites is taken into account, the calculations indicate that, at a concentration of 0.5 nM [³H]WIN 35,065-2, ~50% of the binding is to high-affinity sites and 50% of the binding is

to low-affinity sites. At a ligand concentration of 50 nM, however, 13% of the binding is to high-affinity sites, whereas 87% is to low-affinity sites. Similar increases in specific binding in the presence of 120 mM NaCl are observed under each condition, a result suggesting that [³H]WIN 35,065-2 binding to both high- and low-affinity sites is sodium dependent.

Table 1 indicates the results of a pharmacological characterization of [³H]WIN 35,065-2 binding sites. Several compounds were tested for their potency in inhibiting 50 nM [³H]WIN 35,065-2 binding to predominantly low-affinity sites in rat striatal membranes. GBR 12909, mazindol, and nomifensine are potent inhibitors of ligand binding. The stereospecificity of this binding site is indicated by the relatively weak binding affinity of (+)-cocaine, the dextrorotatory en-

FIG. 4. Effect of NaCl concentration on [³H]WIN 35,065-2 binding. Tissues were incubated with ligand at either 0.5 or 50 nM in 50 mM Tris buffer for 1 h in the presence or absence of 120 mM NaCl. In the absence of NaCl, equimolar N-methyl-D-glucamine was added to the assay tubes to maintain ionic strength. Nonspecific binding was determined by addition of 100 μM (−)-cocaine. Determination of receptor occupancies indicates that at low concentrations of ligand 50% of specific binding is to high-affinity sites, whereas at high concentrations most binding (87%) is to low-affinity sites. The data presented are averages of three assays.

TABLE 1. *Inhibition of [³H]WIN 35,065-2*
binding in striatum

Drug	IC₅₀ (µM)	n
GBR 12909	0.024 ± 0.010	5
Mazindol	0.29 ± 0.044	5
Nomifensine	0.43 ± 0.098	4
WIN 35,065-2	0.84 ± 0.196	3
(−)-Cocaine	1.81 ± 0.408	6
(+)-Cocaine	234.0 ± 34.60	4
(+)-Pseudococaine	304.0 ± 124.0	4
(−)-Pseudococaine	561.0 ± 146.0	4
Citalopram	>1	3
Desipramine	23.2 ± 7.36	3

Inhibition of binding of 50 n*M* [³H]WIN 35,065-2 to striatal membrane preparations by various compounds was measured. Mean ± SE values were calculated for three to six assays as shown.

antiomer of cocaine. However, (+)- and (−)-pseudococaine are equally weak at the [³H]WIN 35,065-2 binding site. Table 1 also shows that desipramine, a selective norepinephrine uptake blocker with nanomolar potency, is a relatively weak inhibitor of [³H]WIN 35,065-2 binding. Citalopram, a potent serotonin uptake blocker, does not appear to inhibit binding at all, a finding suggesting that [³H]WIN 35,065-2 does not bind to serotonin transporters under our conditions.

Table 2 indicates the effect of 6-OHDA lesions of the striatal region on [³H]WIN 35,065-2 binding. The results show that 6-OHDA lesions produced a 53% decrease in dopamine levels in treated animals, relative to those in untreated animals. Large depletions in binding were observed when either high or low concentrations of ligand were used. At 0.5 n*M* [³H]WIN 35,065-2, a concentration at which binding to high- and low-affinity sites each is ∼50% of the total, there was a 67% decrease in specific binding in animals treated with 6-OHDA. At the 50 n*M* concentration of radioligand, at which 13% of specific binding is to high-affinity sites and 87% is to low-affinity sites, there was a 96% reduction in binding in the treated animals. These results could only occur if both high- and low-

TABLE 2. *Loss of [³H]WIN 35,065-2 binding*
following 6-OHDA lesions

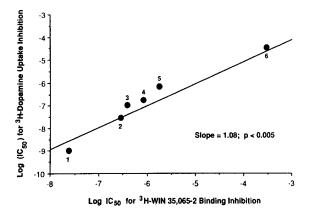| [³H]WIN 35,065-2 (n*M*)	Unlesioned	Lesioned	% decrease
0.5	515 ± 95	169 ± 47	67
50.0	4,518 ± 414	170 ± 35	96

Specific bindings in unlesioned and lesioned groups are expressed as mean ± SEM values (n = 4), in dpm/mg of protein. Lesions and assays were conducted as described in Materials and Methods. Dopamine levels in the lesioned group were 53% below those in the control group (control, 3.25 ± 0.53 pmol/mg of protein). At the low concentration (0.5 n*M*) of ligand, ∼50% of the specific binding is to the high-affinity site, whereas at the high concentration (50 n*M*) of ligand, most binding (87%) is to the low-affinity site.

affinity [³H]WIN 35,065-2 binding sites were significantly associated with the dopamine nerve terminal. Our results also indicated that specific binding was enhanced using the P₂ tissue preparation, an observation suggesting that [³H]WIN 35,065-2 binding sites are concentrated in synaptosomes (data not shown).

Finally, Fig. 5 shows the regression of potency for inhibition of [³H]dopamine uptake on potency for inhibition of [³H]WIN 35,065-2 binding for these compounds (slope = 1.08; p ≤ 0.005). This statistical relationship is consistent with [³H]WIN 35,065-2 binding to the dopamine transporter.

DISCUSSION

[³H]WIN 35,065-2 binding exhibits several similarities with [³H]cocaine binding to dopamine transporters (Kennedy and Hanbauer, 1983; Shoemaker et al., 1985; Madras et al., 1989b), and, thus, these ligands presumably bind to the same site. Both ligands bind to high- and low-affinity sites in striatum. The binding of both ligands is sodium dependent when they are incubated with tissues in Tris buffer. In addition, significant correlations are observed between the potencies of various drugs in inhibiting either [³H]WIN 35,065-2 or [³H]cocaine binding in striatum and their potencies in inhibiting [³H]dopamine uptake (Kennedy and Hanbauer, 1983; Reith et al., 1986; Madras et al., 1989b). These characteristics of [³H]WIN 35,065-2 or [³H]cocaine binding are also similar to those associated with the binding of [³H]mazindol, [³H]nomifensine, and [³H]GBR 12935 (Javitch et al., 1984; Dubocovich and Zahniser, 1985; Janowsky et al., 1986; Anderson, 1987), although these latter compounds are reported to bind to a single site.

Calligaro and Eldefrawi (1987, 1988) found that [³H]cocaine bound to two sites in several brain regions,

FIG. 5. Regression of potency for inhibition of [³H]dopamine uptake in striatum on potency for inhibition of [³H]WIN 35,065-2 binding for several drugs. The compounds shown are (1) GBR 12909, (2) mazindol, (3) nomifensine, (4) WIN 35,065-2, (5) (−)-cocaine, and (6) (+)-pseudococaine. Values for inhibition of dopamine uptake were taken from Anderson (1987) for compounds 1–3 and 5 and from Reith et al. (1986) for compounds 4 and 6.

including striatum, using a sodium phosphate buffer containing 0.32 M sucrose. Our results using Tris buffer suggest that their observation is not dependent on their particular buffer conditions. However, the multiple binding sites for cocaine found outside the striatum by Calligaro and Eldefrawi (1988) could not be shown to be associated with dopaminergic nerve terminals. In contrast, within the striatum, our data from lesion experiments indicate that both sites are associated with dopaminergic nerve terminals. Consistent with this, our data show that both high- and low-affinity [³H]WIN 35,065-2 sites are sodium dependent in Tris buffer. Because the assay conditions used in the present study are similar to those previously used for [³H]mazindol, [³H]nomifensine, and [³H]GBR 12935 binding (Javitch et al., 1984; Dubocovich and Zahniser, 1985; Janowsky et al., 1986; Anderson, 1987), they may facilitate future comparisons between the binding characteristics of these compounds at the dopamine transporter. Shoemaker et al. (1985) also found two binding sites for cocaine in both human putamen and rat striatum under conditions very similar to those used in the present study. However, the high-affinity (0.36 μM) site observed in their study probably corresponds to our low-affinity site, whereas their low-affinity site (26 μM) has not been investigated in this or other studies.

The results of several studies suggest the presence of multiple binding sites or domains on the dopamine transporter. There are the high- and low-affinity sites for cocaine and its analogues (Calligaro and Eldefrawi, 1988; Madras et al., 1989a,b); the significance of these sites is unknown. Other studies report that ligands such as [³H]GBR 12935 bind to a single site and hence may bind to yet another site or domain. Finally, the weak binding of agonists such as dopamine at the transporter suggests a separate site for these compounds, although other explanations have been offered (Anderson, 1987; Zimanyi et al., 1989).

[³H]WIN 35,065-2 may have several advantages over [³H]cocaine in both in vitro and in vivo experiments. The first minor advantage is that it exhibits a consistently higher affinity for the low-affinity site and three- to sevenfold higher affinity for the high-affinity site in in vitro studies than [³H]cocaine (Calligaro and Eldefrawi, 1987, 1988). Under conditions in our laboratory, [³H]WIN 35,065-2 binding also appears to exhibit ~10-15% better specific-to-nonspecific ratios than [³H]cocaine binding under conditions of similar receptor occupancy. [³H]WIN 35,065-2 provides two other major advantages over [³H]cocaine, however. First, it is likely to be more resistant to metabolic or chemical degradation owing to the elimination of the ester linkage between the phenyl and tropane rings. Indeed, in in vivo binding studies, a greater fraction of total binding is specific when [³H]WIN 35,065-2 and [³H]WIN 35,428 are used than when [³H]cocaine is used (Scheffel et al., 1989). Finally, our results suggest that [³H]WIN 35,065-2 does not interact with the serotonin transporter in striatum as cocaine does (Reith

et al., 1985). This finding may be due in part to the observation that cocaine has a higher affinity for the serotonin transporter than for the dopamine transporter, whereas WIN 35,065-2 exhibits equal affinities for both (Reith et al., 1986; Ritz et al., 1987). Indeed, Pitts and Marwah (1988) have shown that serotonergic dorsal raphe neurons are more sensitive to cocaine than ventral segmental or zona compacta dopaminergic neurons. This differential binding affinity to dopamine and serotonin transporters is advantageous for in vitro experiments but especially for in vivo studies involving whole brain receptor binding analyses. Thus, [³H]WIN 35,065-2 binding exhibits important similarities with [³H]cocaine binding while reducing some methodological difficulties characteristic of its use in binding studies.

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