Biochemical Pharmacology, Vol. 51, pp. 563–566, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02208-2

SHORT COMMUNICATION

[^{3}H]WIN 35,428 [2β-Carbomethoxy-3β-(4-fluorophenyl)tropane] Binding to Rat Brain Membranes

COMPARING DOPAMINE CELL BODY AREAS WITH NERVE TERMINAL REGIONS

Nian-Hang Chen, Cen Xu,† Lori L. Coffey† and Maarten E. A. Reith†‡*

*SCHOOL OF BASIC MEDICAL SCIENCES, NANJING MEDICAL UNIVERSITY, NANJING 210029, PEOPLE'S REPUBLIC OF CHINA; AND †DEPARTMENT OF BASIC SCIENCES, UNIVERSITY OF ILLINOIS COLLEGE OF MEDICINE, PEORIA, IL 61656, U.S.A.

ABSTRACT. Potential differences between somatodendritic and axonal dopamine transporters were examined by comparing the binding constants of [^{3}H]WIN 35,428 [2β-carbomethoxy-3β-(4-fluorophenyl)tropane] binding to membranes prepared from the rat ventral mesencephalon, containing A9 and A10 dopamine cell bodies, and from the nucleus accumbens. Saturation analysis of [^{3}H]WIN 35,428 binding, in the presence of compounds to occlude norepinephrine and serotonin transporters, was performed by both the "unlabeled" method (varying unlabeled ligand) and "labeled" method (varying radioligand). The density of binding was substantially lower in the ventral mesencephalon than in the nucleus accumbens, but the binding affinity was only slightly different. Likewise, the differences between the two regions in the inhibitory potency of cocaine and GBR 12909 [1-(2-(di(4-fluorophenyl)-methoxy)-ethyl)-4-(3-phenylpropyl)piperazine] were not substantial. The results suggest that somatodendritic and axonal dopamine transporters in the ventral mesencephalon and nucleus accumbens are not very different as far as their binding domains for uptake blockers such as cocaine and GBR 12909 are concerned. BIOCHEM PHARMACOL 51;4:563–566, 1996.

KEY WORDS. dopamine transporter; WIN 35,428 binding; rat ventral mesencephalon; rat nucleus accumbens; rat striatum

Central dopaminergic neurons release dopamine not only from their axon terminals, but also from their dendrites, and the released dopamine is inactivated by active reuptake into the dopamine neuron [1–3]. In consonance, autoradiographic studies with high-affinity radioligands show the presence of the dopamine transporter not only in dopaminergic nerve terminals but also in cell body areas [4, 5]. However, it is not clear whether axonal and somatodendritic transporters have the same properties. Thus, [^{3}H]dopamine uptake into slices of rat ventral tegmentum is inhibited by various compounds with the same potency as that observed for the inhibition of uptake into NACC§ slices [2]; however, caution is needed when interpreting these results because more recent observations indicate the potential for [^{3}H]dopamine to be taken up into serotonin terminals [3, 6]. The binding of [^{3}H]WIN 35,428 in monkey substantia nigra, measured by autoradiography in coronal brain sections, is inhibited by various blockers with a rank order similar to that in slices from caudate nucleus or putamen [7]; for the caudate-putamen, the IC_{50} values for various blockers in sections correlate closely with values obtained with membrane preparations [8]. However, detailed comparative information on the affinity of compounds for somatodendritic and axonal dopamine transporters is not available in the literature. In the present study, membranes were prepared from the VM, containing A9 and A10 dopamine cell bodies, and from the NACC, and the binding of [^{3}H]WIN 35,428 was studied in the presence of citalopram and desipramine to occlude serotonin and norepinephrine transporters. Saturation analysis was conducted by varying either the nonradioactive WIN 35,428 ("unlabeled" method) or the [^{3}H]WIN 35,428 ("labeled" method), and the inhibitory potencies of cocaine and GBR 12909 were determined.

MATERIALS AND METHODS

Male Sprague–Dawley rats (Harland Sprague–Dawley, Indianapolis, IN) between 8 and 10 weeks of age (225–300 g) were decapitated. For each experiment, the following brain regions were dissected rapidly as described by us previously [3]: the NACC and VM (from six rats, average total tissue weight: 110 mg for NACC and 117 mg for VM) and striatum (from one

‡ Corresponding author: M. E. A. Reith, Ph.D., Department of Basic Sciences, University of Illinois College of Medicine, Box 1649, Peoria, IL 61656. Tel. (309) 671-8545; FAX (309) 671-8403.

§ *Abbreviations:* NACC, nucleus accumbens; VM, ventral mesencephalon; GBR 12909, 1-(2-(di(4-fluorophenyl)-methoxy)-ethyl)-4-(3-phenylpropyl) piperazine; and WIN 35,428, 2β-carbomethoxy-3β-(4-fluorophenyl)tropane.

Received 24 April 1995; accepted 18 September 1995.

rat, average tissue weight 75 mg) (sufficient for 30 binding assays or 10 conditions in triplicate per region). The tissues were homogenized in 2 mL of ice-cold 0.32 M sucrose with a Teflon–glass homogenizer. The pestle was then rinsed with 3 mL of sucrose solution, and this fluid was combined with the original homogenate, which was the starting material for the preparation of the P_2 fraction. All following steps in preparing and assaying P_2 preparations, and analyses of binding data, were as described previously except where indicated [9]. In the present experiments, P_2 pellets from six rats were homogenized with a Teflon–glass homogenizer in 5.5 mL of assay buffer (30 mM sodium phosphate, pH 7.4, at 25°, 0.32 M sucrose), giving on the average 120 μg of protein/150 μL of suspension. The binding assay mixtures contained in a final volume of 0.6 mL approximately 120 μg of protein (delivered by the 150-μL aliquot), 90 nM citalopram for blocking serotonin transporters, and 90 nM desipramine for blocking norepinephrine transporters. Saturation analysis by the unlabeled method involved adding increasing concentrations of unlabeled WIN 35,428 to assays containing a fixed concentration of [^{3}H]WIN 35,428 (2.2 nM for NACC or striatum and 4.5 nM for VM). Unlabeled WIN 35,428 ranged from 0.2 to 40 nM in a total of 8 points. Nonspecific binding was defined with 30 μM cocaine. When the labeled method of saturation analysis was used, increasing concentrations of [^{3}H]WIN 35,428, ranging from 0.3 to 115 nM in a total of 7–9 points, were added along with either 30 μM cocaine or equivalent water volume. The specific activity of the batch of [^{3}H]WIN 35,428 used in this work (Lot No. 2824-220) was determined as described by us previously [10] by performing WIN 35,428 inhibition curves at four different radioligand concentrations, and turned out to be 48.2 dpm/fmol. Because the protein concentrations used in the present binding assays were higher (*ca.* 120 μg/600 μL) than used in our previous studies (*ca.* 25 μg/200 μL), control experiments were carried out with various protein concentrations, and it was found that [^{3}H]WIN 35,428 binding was linear with protein up to 150 μg/600 μL, the highest concentration tested.

RESULTS AND DISCUSSION

Saturable binding of [^{3}H]WIN 35,428 to membranes prepared from the NACC and the VM was best described by a one-site model. Figure 1 shows examples from the unlabeled experiments; data from the labeled analysis were similar. In a region such as the VM where [^{3}H]WIN 35,428 binding is low, saturation analysis is ideally performed with both the labeled and unlabeled method. The former allows a range of radioligand concentrations starting low, which results in increasing values for bound radioactivity as radioligand levels are increased; at the upper end, cost becomes the major limiting factor. The unlabeled method, on the other hand, allows the study of

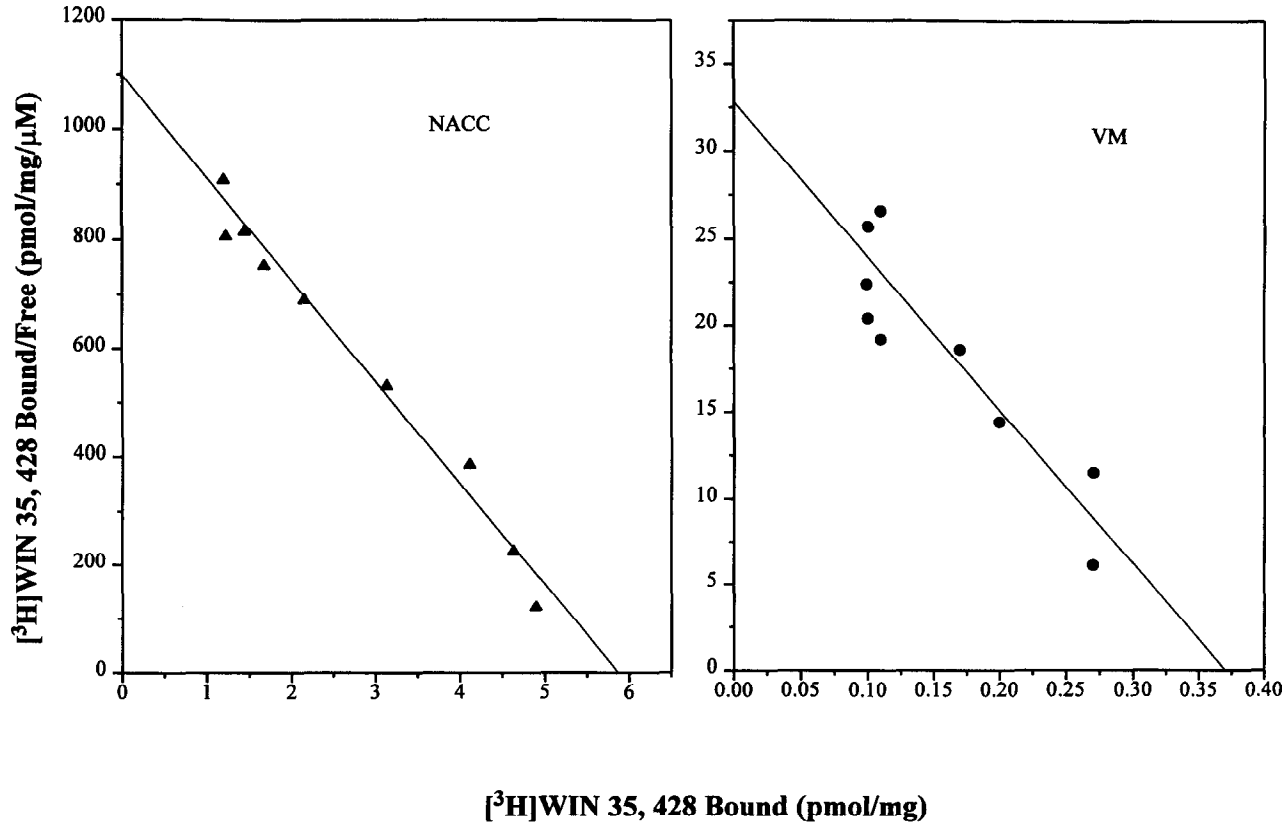

FIG. 1. Saturation analysis by the unlabeled method graphed as Scatchard plots for illustrative purposes. Straight lines represent the best fits chosen by the LIGAND program. A representative experiment is shown that was carried out four times for the NACC and three times for the VM (see Table 1 for averages of all unlabeled and labeled results together).

relatively higher concentrations of nonradioactive ligand but requires a substantial labeling at the fixed radioligand concentration chosen as the starting point (well below the K_d). Under the present conditions for the VM, within the constraints of available brain tissue, the lowest possible radioligand concentration was 0.3 nM with the labeled method (on the average 225 dpm specific binding per assay) and the fixed radioligand concentration was 4.5 nM with the unlabeled method (750 dpm). These low, but detectable, binding values probably underlie the greater variation in the binding data collected for the VM as compared with the NACC. Because the specific activity of the radioligand impacts on the estimation of the binding constants, especially with the labeled method [11], we addressed this experimentally for the batch of radioligand used in this study. We used the competition method for measuring specific activity, as described previously [10].

The results taken together indicated a slightly elevated K_d for [^{3}H]WIN 35,428 binding in the VM ($P < 0.05$, two-tailed t-test) (Table 1). Cocaine also appeared to have a slightly higher K_i in the VM as compared with the NACC ($P < 0.05$, one-tailed t-test); the Hill numbers were close to unity in both regions. The results obtained for GBR 12909 did not indicate a regional difference in the same direction as for the cocaine-like compounds; if indeed more observations would substantiate the tendency for GBR 12909 to be a stronger, rather than weaker, inhibitor in the VM, it would support the notion of different binding domains involved in GBR- and cocaine-like compounds. The Hill numbers for GBR 12909 tended to be somewhat higher than unity, but this did not reach statistical significance (one-sample, Student's t-test) (Table 1). Overall, the differences in potencies, between regions, were not substantial, suggesting that somatodendritic and axonal dopamine transporters have comparable affinities for cocaine- and GBR-related compounds. In this context, it is of interest that i.v. administered cocaine may be metabolized relatively faster in the substantia nigra of primate brain as compared with other brain regions including caudate-putamen and NACC [12].

TABLE 1. Binding of [^{3}H]WIN 35,428, cocaine, and GBR 12909 to membranes prepared from NACC and VM

	NACC	VM
[^{3}H]WIN 35,428		
K_d (nM)	5.2 ± 0.2 (8)	7.7 ± 1.2* (6)
B_{max} (pmol/mg)	3.46 ± 0.44 (8)	0.53 ± 0.16† (6)
Cocaine		
K_i (nM)	47.4 ± 0.98 (3)	66.4 ± 7.82‡ (3)
Hill	0.96 ± 0.03 (3)	0.99 ± 0.08 (3)
GBR 12909		
K_i (nM)	2.8 ± 0.36 (3)	1.9 ± 0.54 (2)
Hill	1.3 ± 0.29 (3)	1.2 ± 0.12 (2)

Values are means ± SEM (=0.5 × range where $N = 2$). The number indicated between parentheses denotes the number of independent membrane preparations, with each preparation consisting of pooled tissue from six rats. The K_d and B_{max} values from the unlabeled and labeled experiments were combined.

*$P < 0.05$ compared with the same variable in NACC (Student's t-test, two-tailed).

† $P = 0.0001$ compared with the same variable in NACC (Student's t-test, two-tailed).

‡ $P < 0.05$ compared with the same variable in NACC (Student's t-test, one-tailed with alternative hypothesis that K_i is higher in VM).

The present results would suggest that such a diminished activity of cocaine is not compensated for by an increased affinity of cocaine towards somatodendritic dopamine transporters; rather, the slightly reduced affinity would accomplish the opposite. In the present *in vitro* experiments at 0–4°, cocaine is not likely to be metabolized as no metabolism of [^{3}H]cocaine was found with rodent brain membranes at 21° [13].

The B_{max} of [^{3}H]WIN 35,428 binding sites was substantially (7-fold) lower in the VM than in the NACC (Table 1). Most likely, this is partly due to tissue surrounding the A9 and A10 somatodendritic regions that does not contain dopamine transporters, because autoradiographic studies show only an approximately 2-fold difference in dopamine transporter labeling between the ventral tegmental area/substantia nigra (combined in our dissection of the VM) and the NACC. Our attempts to measure [^{3}H]dopamine uptake into VM synaptosomes in the presence of 90 nM citalopram and desipramine were unsuccessful. The uptake was much lower than anticipated from the reduced density of [^{3}H]WIN 35,428 sites, indicating, for that region, a relatively lower affinity for dopamine, a lower dopamine uptake velocity, or an increased sensitivity to citalopram/desipramine. The addition of these inhibitors was necessary because [^{3}H]dopamine can be taken up into serotonergic terminals in dopamine cell body areas [3, 6], and dopamine has a high affinity for the norepinephrine transporter, in fact higher than norepinephrine itself as described for brain preparations by Coyle and Snyder [14] and the cloned norepinephrine transporter by Pacholczyk *et al.* [15].

In addition to the lack of differences in WIN 35,428 binding between cell body and terminal dopamine transporters observed in this study, the affinity of the transport complex for WIN 35,428 was similar in the two different terminal areas of the mesolimbic pathway (the NACC), and the nigrostriatal pathway (the striatum). Thus, the K_d of striatal [^{3}H]WIN 35,428 binding was 6.8 ± 1.5 nM (mean ± SEM for three independent preparations, each derived from one rat) when measured under the present conditions, close to the value observed under the same experimental conditions in the NACC (Table 1). The B_{max} was 9.7 ± 1.2 pmol/mg protein, as expected somewhat higher than that found for the NACC. Other reports have described generally similar properties of dopamine transporters in the striatum and NACC [16, 17]. All data taken together with the present results suggest that the dopamine transporter is similar in the VM, NACC, and striatum.

The observation of Einhorn *et al.* [18] that cocaine is much weaker in suppressing dopamine cell firing when administered iontophoretically into the ventral tegmental area than when injected intravenously, as well as our own findings showing that focal application of cocaine into the ventral tegmental area is less effective in enhancing extracellular dopamine than application into the NACC (manuscript in preparation), are probably not the result of a lower affinity of cocaine for somatodendritic as compared with axonal dopamine transporters. Rather, the present study suggests that other factors should be considered. One is the lower density of dopamine uptake

sites in the ventral tegmental area as compared with the NACC (see above), and another factor is the existence of an inhibitory NACC-ventral tegmental area feedback pathway [18]. The present study does not rule out the possibility that there are regionally dependent differences in monoamine transport functioning beyond the level of substrate, blocker, and Na^+ recognition.

This research was supported by USPHS Grant DA 08379 (M.E.A.R.). The work was made possible by a visit from Dr. Nian-Hang Chen, which was generously permitted by the School of Basic Medical Sciences, Nanjing Medical University, Nanjing, People's Republic of China.

References

1. Arbilla S and Langer SZ, Influence of monoamine oxidase inhibition on the release of ^{3}H-dopamine elicited by potassium and by amphetamine from the rat substantia nigra and corpus striatum. *Naunyn Schmiedebergs Arch Pharmacol* **311:** 45–52, 1980.
2. Beart PM and McDonald D, Neurochemical studies of the mesolimbic dopaminergic pathway: Somatodendritic mechanisms and GABAergic neurones in the rat ventral tegmentum. *J Neurochem* **34:** 1622–1629, 1980.
3. Chen N-H and Reith MEA, [^{3}H]Dopamine and [^{3}H]serotonin release *in vitro* induced by electrical stimulation in A_9 and A_{10} dopamine regions of rat brain: Characterization and responsiveness to cocaine. *J Pharmacol Exp Ther* **267:**379–389, 1993.
4. Javitch JA, Strittmatter SM and Snyder SH, Differential visualization of dopamine and norepinephrine uptake sites in rat brain using [^{3}H]mazindol autoradiography. *J Neurosci* **5:** 1513–1521, 1985.
5. Mennicken F, Savasta M, Peretti-Renucci R and Feuerstein C, Autoradiographic localization of dopamine uptake sites in the rat brain with ^{3}H-GBR 12935. *J Neural Transm* **87:** 1–14, 1992.
6. Kelly E, Jenner P and Marsden CD, Evidence that [^{3}H]dopamine is taken up and released from nondopaminergic nerve terminals in the rat substantia nigra *in vitro*. *J Neurochem* **45:** 137–144, 1985.
7. Kaufman MJ, Spealman RD and Madras BK, Distribution of cocaine recognition sites in monkey brain: I. *In vitro* autoradiography with [^{3}H]CFT. *Synapse* **9:** 177–187, 1991.
8. Canfield DR, Spealman RD, Kaufman MJ and Madras BK, Autoradiographic localization of cocaine binding sites by [^{3}H]CFT ([^{3}H]WIN 35,428) in the monkey brain. *Synapse* **6:** 189–195, 1990.
9. Coffey LL and Reith MEA, [^{3}H]WIN 35,428 binding to the dopamine uptake carrier. I. Effect of tonicity and buffer composition. *J Neurosci Methods* **51:** 23–30, 1994.
10. Wiener HL and Reith MEA, Determination of radioligand specific activity using competition binding assays. *Anal Biochem* **207:** 58–62, 1992.
11. Reith MEA and Selmeci G, 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.
12. Madras BK and Kaufman BJ, Cocaine accumulates in dopamine-rich regions of primate brain after i.v. administration: Comparison with mazindol distribution. *Synapse* **18:** 261–275, 1994.
13. Reith MEA, Sershen H and Lajtha A, Saturable [^{3}H]cocaine binding in central nervous system of mouse. *Life Sci* **27:** 1055–1062, 1980.
14. Coyle JT and Snyder SH, Catecholamine uptake by synaptosomes in homogenates of rat brain: Stereospecificity in different areas. *J Pharmacol Exp Ther* **170:** 221–231, 1969.
15. Pacholczyk T, Blakely RD and Amara SG, Expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter. *Nature* **350:** 350–354, 1991.
16. Berger P, Elsworth JD, Arroyo J and Roth RH, Interaction of [^{3}H]GBR 12935 and GBR 12909 with the dopamine uptake complex in nucleus accumbens. *Eur J Pharmacol* **177:** 91–94, 1990.
17. Boja JW and Kuhar MJ, [^{3}H]Cocaine binding and inhibition of [^{3}H]dopamine uptake is similar in both the rat striatum and nucleus accumbens. *Eur J Pharmacol* **173:** 215–217, 1989.
18. Einhorn LC, Johansen PA and White FJ, Electrophysiological effects of cocaine in the mesoaccumbens dopamine system: Studies in the ventral tegmental area. *J Neurosci* **8:** 100–112, 1988.