Molecular Neurobiology

ISSN0893-7648/95/11(1–3): 111–119/$5.80

Rat Mesencephalic Neuronal Cells Cultured for Different Periods as a Model of Dopamine Transporter Ontogenesis

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Abstract

Ventral mesencephalic neurons contained only low-affinity and sodium-independent binding sites of [^{3}H]WIN 35,428 (marker of dopamine transporter) during the first 10 d in primary cultures. These sites were present in cytosol, and they are not very probably related to dopamine transporter. After 12 d in culture, membrane-bound, high-affinity, and sodium-dependent [^{3}H]WIN 35,428 binding sites were detected. In membranes prepared from cells 14 d in culture, cocaine displaced [^{3}H]WIN 35,428 binding with similar potency to that in striatal membranes of adult rat brain. The high-affinity [^{3}H]WIN 35,428 binding sites in mesencephalic neuronal cell cultures are very probably related to dopamine transporter. The development of high-affinity [^{3}H]WIN 35,428 binding sites in neurons cultured for different time periods could be a useful model of dopamine transporter ontogenesis.

Index Entries: Dopamine transporter; ontogenesis; primary cultures; [^{3}H]WIN 35,428 binding.

Introduction

Dopamine uptake is inhibited by series of agents that bind with a high-affinity to the dopamine transporter. The most efficient inhibitors of dopamine uptake are cocaine and its derivatives, diphenylpiperazine compounds (GBR 12909), mazindol, and so on. Some of them are useful dopamine transporter ligands (e.g., cocaine derivative [^{3}H]WIN 35,428) (Kennedy and Hanbauer, 1983; Madras et al., 1989a,b; Ritz et al., 1990). Recently, the dopamine transporter complementary DNA has been cloned and expressed in *Xenopus oocytes* and COS cells (Kilty et al., 1991; Shimada et al., 1991; Boja et al., 1992). In transfected COS cell membranes, [^{3}H]WIN 35,428 binding had a similar pharmacological profile as that in striatal membranes (Shimada et al., 1991; Boja et al., 1992), indicating that dopamine uptake and [^{3}H]WIN 35,428 recognition sites were encoded by the same cDNA.

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It is well known that high-affinity dopamine uptake is present in primary mesencephalic neuronal cell cultures prepared from rat embryos (Prochiantz et al., 1979; Cerruti et al., 1991; Grilli et al., 1991). Specific binding of [^{3}H]cocaine and [^{3}H]mazindol was found in these intact cell cultures cultured for 5 d, and the binding sites were present in cytosol (Grilli et al., 1991). It was not clear whether these sites are related to dopamine transporter.

In this study, we used [^{3}H]WIN 35,428 as a ligand to characterize its binding sites in intact mesencephalic neurons cultured for 5 d. We found that these sites are not related to dopamine transporter. In the following experiments, we extended the time of cultivation until 20 d. The second, high-affinity [^{3}H]WIN 35,428 binding site appeared after 12 d in culture, and it is related to the dopamine transporter. It is proposed that measurement of [^{3}H]WIN 35,428 binding in mesencephalic neuronal cells cultured far different periods would be a useful model for the studies of dopamine transporter ontogenesis.

Materials and Methods

Drugs and Radioactive Materials

[^{3}H]WIN 35,428 and [^{3}H]dopamine were used from NEN (Boston, MA). The source of GBR 12909 and WIN 35,428 was Research Biochemicals, Inc. (Natick, MA); cocaine·HCl methane sulfonate was obtained from Merck, Sharp, and Dohme (St. Louis, MO).

Preparation of Cell Cultures

Primary cultures of mesencephalic neurons were prepared from 14-d-old embryos (precisely timed pregnant Sprague-Dawley rats; Zivic-Miller, PA) as described previously (Valchář and Hanbauer, 1993). The rostral portion of the mesencephalon was dissected, and the tissue was mechanically dissociated. Cells were centrifugated (100*g* for 3 min), resuspended in complete medium, and cultured in multiwell plates (16 mm diameter; Costar, Cambridge, MA) or in Petri dishes (90 mm diameter) that previously were coated with poly-D-lysine (15 µg/mL, 53,000 mol wt, Sigma, St. Louis, MO). Cells were plated (40,000 cells/cm^2) and cultured for 5–20 d at 37°C in an atmosphere of 95% air and 5% CO_2 saturated with H_2O. The culture medium was composed of a 1:1 mixture of Modified Essential Medium (Gibco BRL, Gaithersburg, MD) and Nutrient Mixture F12 (Gibco BRL), supplemented with 15% horse serum (Hyclone Laboratories, Inc., Logan, UT), 6 mg/mL glucose, 2 m*M* glutamine (Gibco BRL), and 0.5 U/mL penicillin-G and 0.5 mg/mL streptomycin. To inhibit glial cell growth, cytosine-β-D-arabinofuranoside (Sigma) in a final concentration of 1 µ*M* was added on the 5th d in culture. After the 5th d in culture, one quarter of culture medium was replaced each 48 h.

Membrane Preparation of Striata and Mesencephalic Cell Cultures

Striata were dissected from brains of adult male rats (Sprague-Dawley, Zivic Miller, 150–200 g). The tissue was homogenized by Polytron (15 s; setting 5) in 20 vol (w/v) of 50 m*M* Tris-HCl buffer (pH 7.4 at 4°C) and centrifuged at 38,700*g* at 4°C for 20 min. The resulting pellet was resuspended in 40 vol of the buffer, and the procedure was repeated twice. Protein concentration was determined after the last centrifugation (Lowry et al., 1951), and was adjusted to 1.0 mg/mL.

[^{3}H]WIN 35,428 Binding to Striatal Membranes

Modified method of Madras et al. (1989b) was used. Membranes were incubated in Tris-HCl buffer (pH 7.4) in the presence of 120 m*M* NaCl and 4 n*M* [^{3}H]WIN 35,428 in a total volume of 0.5 mL (0.1 mg of protein/sample) at 0–4°C for 2 h. Incubation was stopped by vacuum filtration through Whatman GF/C filters previously soaked with 0.1% polyethyleneimine. Each tube was rinsed. The filters

were washed three times with 4 mL of ice-cold 50 m*M* Tris-HCl buffer, and incubated overnight in 10 mL of scintillation cocktail (Ready Safe, Beckman). Thereafter, radioactivity was measured with Beckman LS5000 TD scintillation spectrometer. Nonspecific binding was measured in the presence of 10 μ*M* GBR 12909.

Cell Culture Membrane Preparation and [^{3}H]WIN 35,428 Binding

Cells cultured in Petri dishes (diameter 90 mm) were washed three times with 3 mL of PBS to remove culture medium. Cells were removed from the bottom of Petri dishes and centrifuged at 38,700*g* at 4°C for 20 min. Pellet was suspended in 40 vol (w/v) 50 m*M* Tris-HCl buffer (pH 7.4 at 4°C) and centrifuged at 38,700*g*. Further steps in membrane preparation and [^{3}H]WIN 35,428 binding were identical to those in striatal membranes.

[^{3}H]WIN 35,428 Binding and [^{3}H]Dopamine Uptake to Intact Cells

The procedure was described previously (Valchář and Hanbauer, 1993). Cells in each well of a multiwell plate were washed three times with 0.5 mL PBS buffer containing Ca^{2+} and Mg^{2+} (Dulbecco and Vogt formulation; Quality Biological Inc., Gaithersburg, MD), and then were incubated in 200 μL of the same buffer containing 4 n*M* [^{3}H]WIN 35,428 or 50 n*M* [^{3}H]dopamine. The binding was terminated by removing the incubation solution and rinsing the cells with ice-cold incubation buffer. The cells were lysed and transferred from the wells into scintillation vials using 0.25 mL of 0.2*N* NaOH containing 0.2% Triton X-100. The wells were rinsed with an equal volume of 0.2*N* HCl containing 0.2% Triton X-100, and the rinses were added to the vials. To the combined solutions, 10 mL of liquid scintillation cocktail were added, and the radioactivity was measured using scintillation spectrometry. The nonspecific binding was determined in the presence of 50 μ*M* GBR 12909.

Data Analysis

The IC_{50} values were calculated by regression analysis (least-square method) of the specific [^{3}H]WIN 35,428 binding. The LIGAND program was used for the estimation of the density of sites (B_{max}) and dissociation constant (K_d) by iterative nonlinear curve fitting (Munson and Rodbard, 1980). One- and two-component binding models were compared using root mean square error of each fit and the F-test.

Results

In cells cultured for 5 d, the [^{3}H]WIN 35,428 binding sites were saturated in a very high concentration of the ligand and only one low-affinity binding site was found (Fig. 1). K_d value in this experiment was 371,000 n*M* (in striatal membranes was 34 n*M*). In these cells, GBR 12909 displaced [^{3}H]WIN 35,428 in much higher concentrations in comparison to striatal membranes. In the case of cocaine displacement, the difference was even higher (Fig. 2). We compared the effects of all available dopamine uptake inhibitors and agents with different molecular mechanisms on [^{3}H]WIN 35,428 binding both in intact mesencephalic neuronal cell cultures and striatal membranes and on [^{3}H]dopamine uptake in intact mesencephalic neuronal cell cultures (*see* in more detail, Valchář and Hanbauer, 1993). No correlation in the drug-inhibitory effects between [^{3}H]WIN 35,428 binding to mesencephalic neuronal cell cultures and striatal membranes was found. Also, no relationship of the drug-inhibitory effects on [^{3}H]dopamine uptake and [^{3}H]WIN 35,428 binding to intact mesencephalic neuronal cell cultures existed, but a good correlation (r = 0.779) appeared between the drug inhibition of [^{3}H]dopamine uptake to mesencephalic neuronal cell cultures and [^{3}H]WIN 35,428 binding in striatal membranes. In mesencephalic neuronal cells cultured for 5 d, other experiments showed that the binding was reversible, sodium-independent, and no

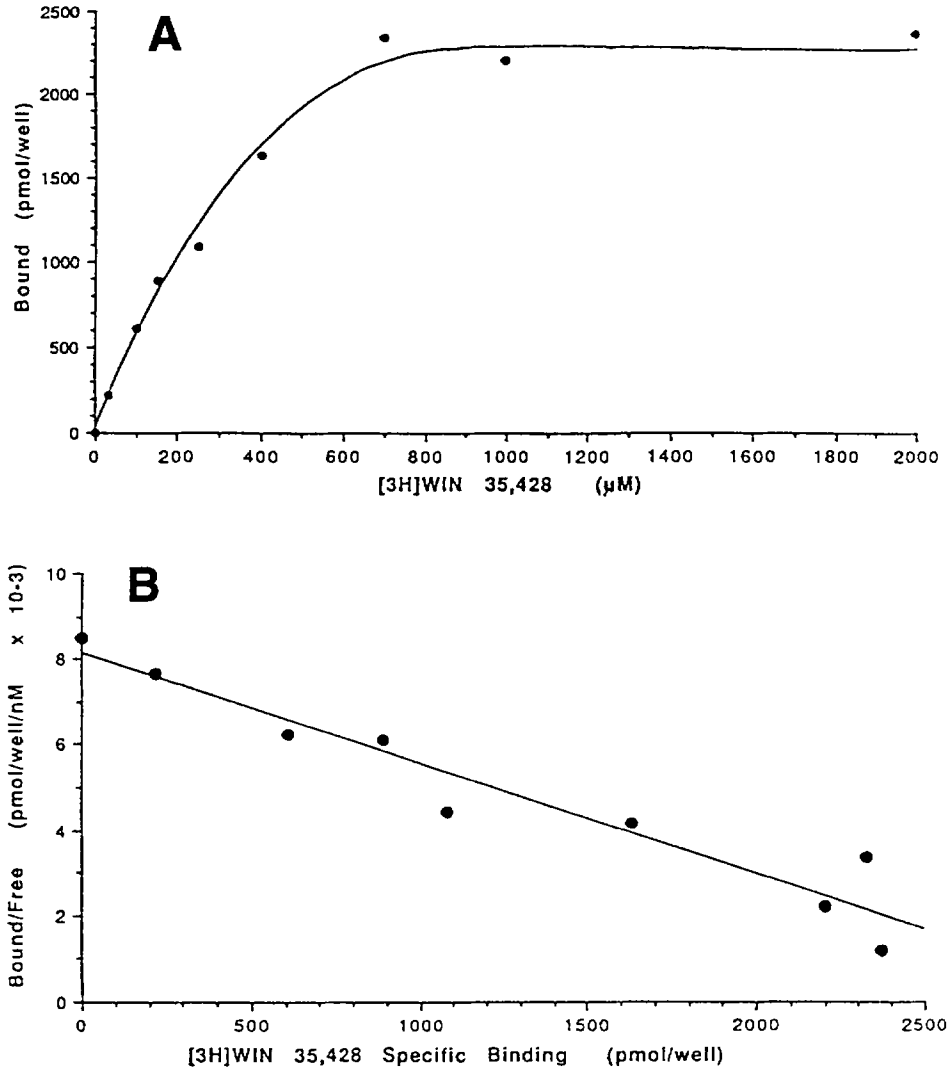

Fig. 1. **(A)** Saturation curve for [^{3}H]WIN 35,428 binding in mesencephalic neuronal cells cultured for 5 d. In saturation experiments, to 4 n*M* [^{3}H]WIN 35,428 was added nonlabeled WIN 35,428 to obtain final ligand concentration ranging from 50–2000 μ*M*. Each point is the mean of two values. **(B)** Scatchard analysis of data from (A) gives a K_d of 371,000 and B_{max} 3.07 pmol/well. For mean data of K_d values, *see* Table 1.

specific binding was measurable in membranes prepared from these cells.

Taken together, the low-affinity binding of [^{3}H]WIN 35,428 in mesencephalic neuronal cells cultured for 5 d, which is present in cytosol, has no relationship to dopamine transporter. Because dopamine transporter is present in these cells, density of its binding sites is not sufficient to be measurable using the [^{3}H]WIN 35,428 method.

In intact cells cultured for 20 d, [^{3}H]WIN 35,428 binding sites were saturated in much lower concentration of the ligand than in mesencephalic cells cultured for 5 d (Fig. 3A). Scatchard analysis of these experiments showed that in cells cultured for 5 d, only one low-affinity binding site existed (Fig. 3B), but in cells cultured for 20 d, it distinguished both high- and low-affinity binding sites of [^{3}H]WIN 35,428 (Fig. 3C).

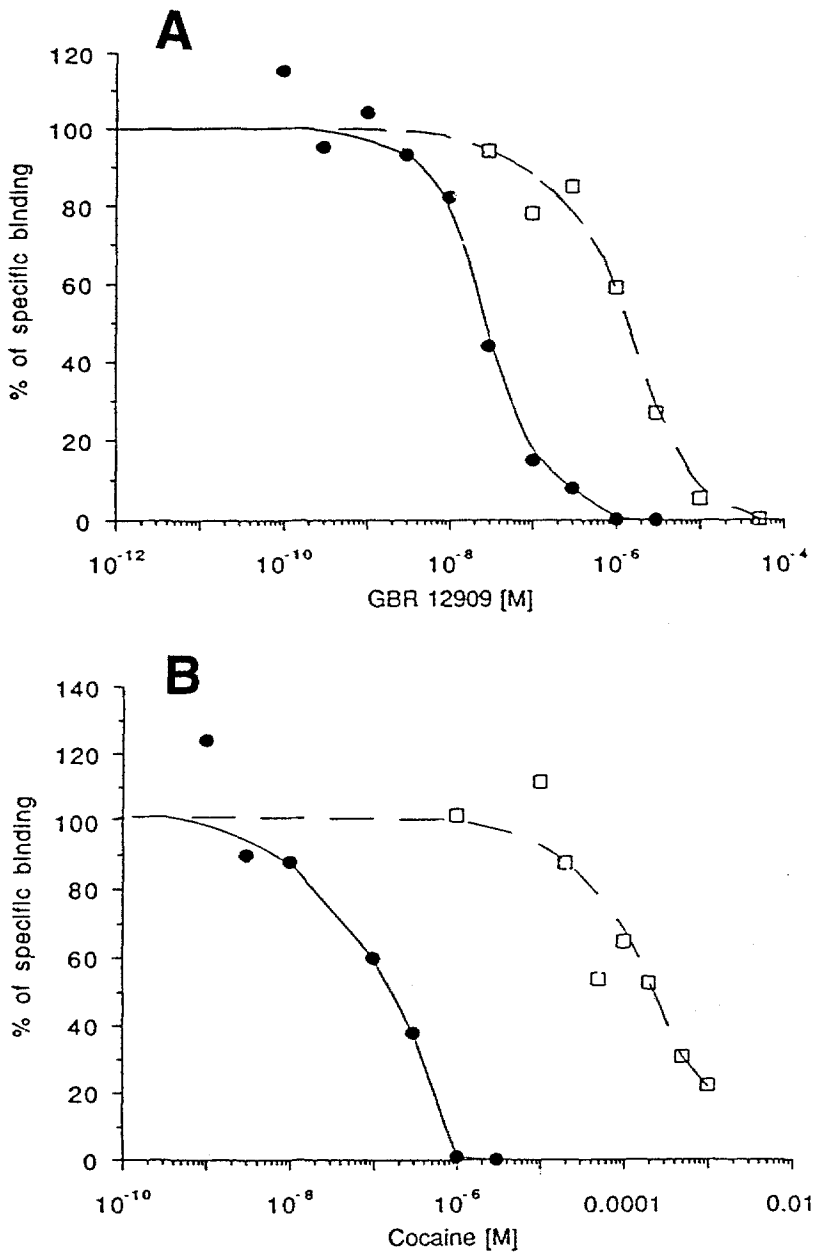

Fig. 2. Displacement of 4 n*M* [^{3}H]WIN 35,428 specific binding by GBR 12909 **(A)** and cocaine **(B)** in striatal membranes of adult rats (black circles) or in intact mesencephalic neuronal cells cultured for 5 d (open squares). Each point is the mean of two values.

The development of [^{3}H]WIN 35,428 binding affinities in intact mesencephalic neuronal cells as a function of time in culture is summarized in Table 1. The high-affinity binding sites were detectable in mesencephalic neuronal cells since 12 d in culture. The K_d value of low-affinity binding sites was not changed during the cultivation.

The binding of ligands to neurotransmitter transporters is Na^+-dependent. This feature

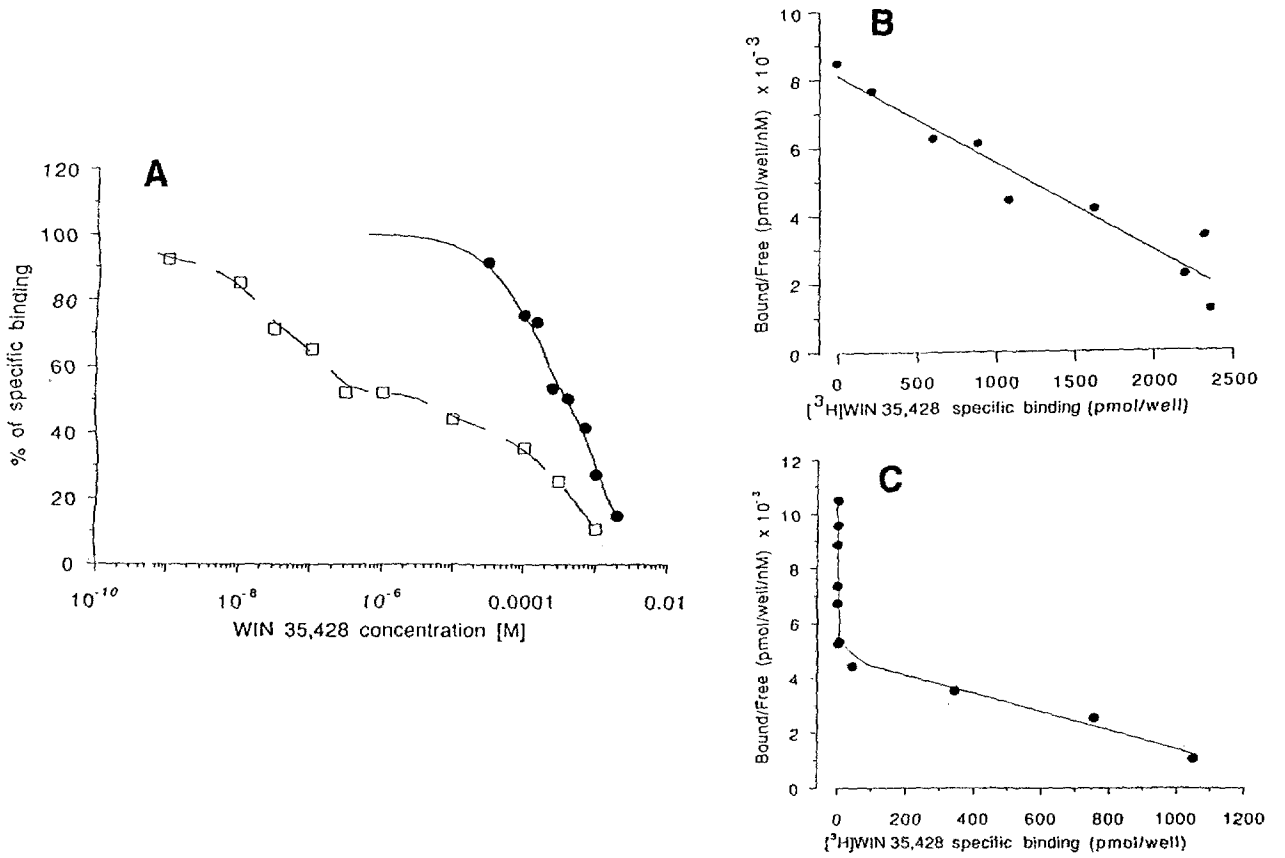

Fig. 3. **(A)** Saturation curves of [^{3}H]WIN 35,428 specific binding to intact mesencephalic neurons cultured for 5 d (black circles) or 20 d (open squares). Final concentrations of the ligand ranged from 4 n*M* to 2 m*M* (for details, see Fig. 1). **(B)** Scatchard analysis of the saturation curve obtained from neurons cultured for 5 d. **(C)** Scatchard analysis of the saturation curve from neurons cultured for 20 d.

Table 1
Affinity of [^{3}H]WIN 35,428 Binding Sites in Primary Mesencephalic Neurons Cultured for Different Periods[a]

	n	K_{d1}, n*M*	K_{d2}, n*M*
5 d	5	n.d.	138,000 ± 22,000
7 d	1	n.d.	151,000
8 d	4	n.d.	170,000 ± 28,000
10 d	3	n.d.	119,000 ± 11,000
12 d	2	77.3	141,000
14 d	2	25.3	80,000
20 d	3	43.0 ± 7.7	193,000 ± 61,000

n.d.—Not detectable.
[a]Means ± SEM are given when $n > 2$ (*n*—number of independent experiments).

was not found in [^{3}H]WIN 35,428 binding in intact mesencephalic neurons cultured for 5 d (Fig. 4A). However, in intact cells cultured and incubated for 14 d in the presence of 120 m*M* NaCl, both high-affinity (K_{d1}= 68 n*M*) and low-affinity (K_{d2}= 124,000 n*M*) binding sites were determined (Fig. 4B), but in the absence of Na^+, only the low-affinity binding site (K_d= 116,000 n*M*) was detected. In membranes prepared from mesencephalic neurons cultured for 14 d, the binding of [^{3}H]WIN 35,428 was of high-affinity (K_d = 45 n*M*) and sodium-dependent (Fig. 5).

Cocaine displaced [^{3}H]WIN 35,428 binding in intact mesencephalic neurons cultured for 14 d in lower concentrations than it did in intact cells

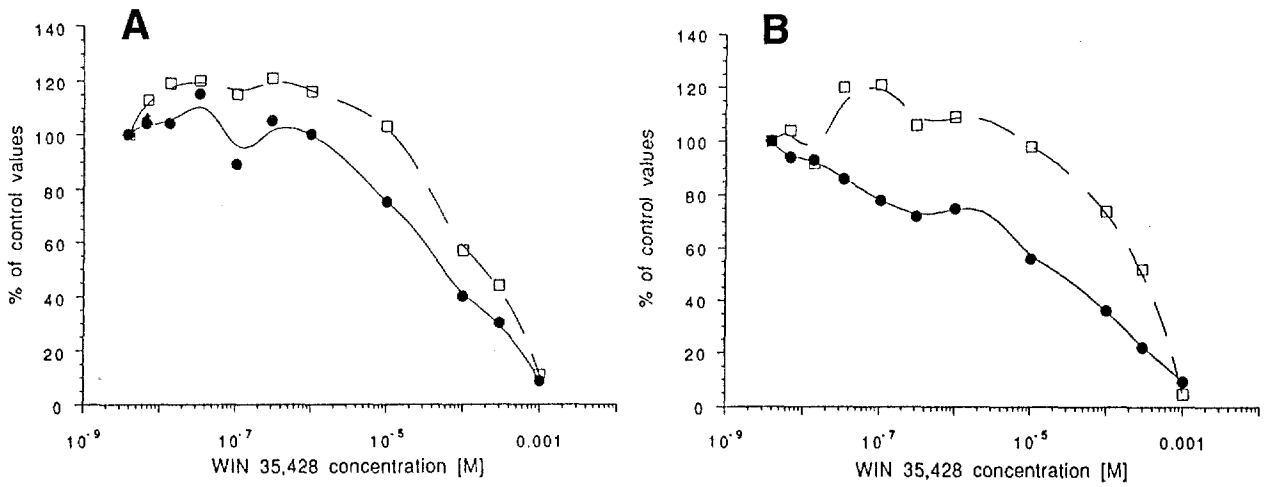

Fig. 4. Sodium-dependency of [^{3}H]WIN 35,428 binding to intact mesencephalic neurons being in culture for 5 d **(A)** or 14 d **(B)**. 120 m*M* NaCl was present (black circles) or absent (open squares) in the incubation medium. In the Na^+-free buffer, NaCl was replaced by choline chloride in the same concentration. Final concentrations of the ligand ranged from 4 n*M* to 1 m*M* (for details, *see* Fig. 1). Each point represents the mean of two determinations in triplicate.

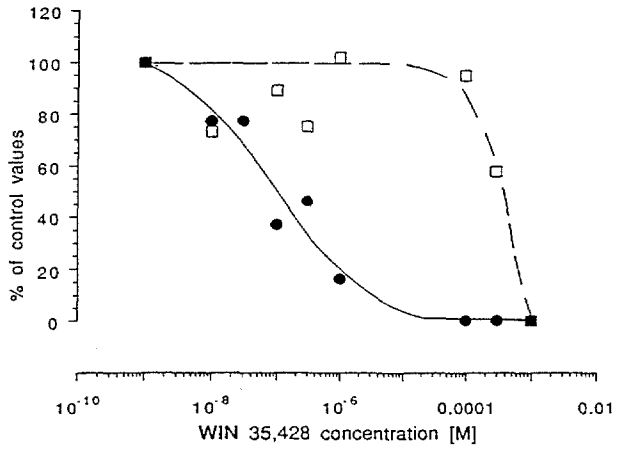

Fig. 5. Sodium dependency of [^{3}H]WIN 35,428 binding to membranes prepared from mesencephalic neurons cultured for 14 d. Membranes were incubated in the presence (black circles) or absence (open squares) of 120 m*M* NaCl. Final concentrations of the ligand ranged from 4 n*M* to 1 m*M* (for details, *see* Fig. 1). Each point is the mean of two determinations in triplicate.

Table 2
Comparison of [^{3}H]WIN 35,428 Binding Inhibition by Cocaine in Membrane Preparations and in Mesencephalic Neurons Cultured for 5 or 14 D[a]

	Cocaine
Striatal membranes	196
Membranes from CVMN[b] cultured for 14 d	193
Intact CVMN cultured for 5 d	340,000
Intact CVMN cultured for 14 d	10,900

[a]The inhibition is expressed in IC_{50} values (n*M*).
[b]CVMN; cultures of ventral mesencephalic neurons.

cultured for 5 d (Table 2). In membranes prepared from mesencephalic neurons cultured for 14 d, cocaine displaced [^{3}H]WIN 35,428 binding practically in the same concentrations as in striatal membranes from adult rat (Table 2).

Conclusion

Our experiments showed that the high-affinity binding of [^{3}H]WIN 35,428 to intact cells cultured for different periods was detectable since 12 d of cultivation. The dissociation constant of the high-affinity binding of the ligand is in the nanomolar range of concentration and its value is comparable to the K_d in striatal

Table 3
Comparison of [^{3}H]WIN 35,428 Binding Site Characteristics in Mesencephalic Neurons Cultured for 5 D and for 14 D (High-Affinity Site) and in Striatal Membranes Prepared from Adult Rats

	Cells cultured for 5 d	Cells cultured for 14 d, high-affinity site	Striatal membranes
K_d (n*M*)	138,000	25.3	34.0
Effective concentrations of dopmine uptake inhibitors	μ*M*	n*M*	n*M*
Sodium dependence	No	Yes	Yes
Location	Cytosol	Membrane	Membrane

membranes. (Table 3). The high-affinity binding was sodium-dependent, and it was present in membranes of the cells. Finally, dopamine uptake inhibitors displaced the membrane-bound [^{3}H]WIN 35,428 binding in the nanomolar concentration range, as it did in striatal membranes. These findings make possible the conclusion that these sites belong to DA transporter. It is evident that cytosolic [^{3}H]WIN 35,428 binding sites in cells cultured for 5 d have different properties and very probably, they are not related to dopamine transporter.

Because it is possible to observe the increase of the high-affinity [^{3}H]WIN 35,428 binding in mesencephalic neuronal cell culture during the cultivation, this approach could be useful in the study of dopamine transporter ontogenesis.

Acknowledgment

The authors thank A. G. Wright, Jr. for his technical assistance. M. Valchář was supported by a research fellowship from ICI Pharmaceuticals, Wilmington, DE, and by a grant from the Scottish Rite Schizophrenia Research Program, Northern Masonic Jurisdiction.

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