Journal of Neurochemistry
Raven Press, Ltd., New York
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Characterization of [^{3}H]Dopamine Uptake Sites and [^{3}H]Cocaine Recognition Sites in Primary Cultures of Mesencephalic Neurons During In Vitro Development

Mariagrazia Grilli, A. Gilbert Wright, Jr., and Ingeborg Hanbauer

Laboratory of Chemical Pharmacology, National Heart, Lung, and Blood Institute, Bethesda, Maryland, U.S.A.

Abstract: [^{3}H]Dopamine uptake and [^{3}H]cocaine binding sites were studied in primary cultures of ventral mesencephalon from 14-day-old rat embryos. Specific binding sites for [^{3}H]cocaine and [^{3}H]mazindol were detected only in intact cell cultures of ventral mesencephalon, and were absent in sonicated, washed membranes prepared from these cell cultures. [^{3}H]Cocaine was not taken up by the cells through an active transport process because [^{3}H]cocaine binding occurred also at 4°C. Moreover, the possibility of [^{3}H]cocaine entering the cells by passive diffusion and ion trapping was also excluded because extensive washing failed to remove [^{3}H]cocaine from the cells. [^{3}H]Cocaine binding was reduced to 6% of control when cells were permeabilized with streptolysin O (0.2 U/ml, 5 min). Taken together, these results suggest that in cultured mesencephalic neurons, [^{3}H]cocaine may enter the cell by passive diffusion and then be sequestered by a cytosolic compartment that is lost in the process of permeabilization or sonication and washing of membrane preparations. Permeabilization of cultured neurons failed to alter the storage of [^{3}H]dopamine. When cells were permeabilized with streptolysin O (0.2 U/ml; 5 min) after [^{3}H]dopamine was taken up, [^{3}H]dopamine was retained by the cells and did not leak into the incubation medium, indicating that [^{3}H]dopamine was stored in sites that could not pass through the perforated membranes. In contrast, [^{3}H]dopamine uptake into already permeabilized cells was reduced by 33%, suggesting that a cytosolic protein that had leaked out may play a functional role in the uptake process. In contrast to striatal membrane preparations of adult rats, [^{3}H]cocaine binding in intact mesencephalic cell cultures was Na^+ independent. The expression of [^{3}H]dopamine uptake and [^{3}H]cocaine binding sites appeared to be developmentally linked to neuritic outgrowth, supporting the view that cocaine binding sites may be closely associated with the dopamine transporter. **Key Words:** Dopamine uptake—[^{3}H]Cocaine binding sites—Primary cultures—Ventral mesencephalon. **Grilli M. et al.** Characterization of [^{3}H]dopamine uptake sites and [^{3}H]cocaine recognition sites in primary cultures of mesencephalic neurons during in vitro development. *J. Neurochem.* **56**, 2108–2115 (1991).

Many central stimulants inhibit catecholamine reuptake in the CNS. The understanding of the molecular mechanism underlying the presynaptic reuptake of catecholamines or the interaction of uptake blockers with the transmitter transporter, however, is still limited. Dopamine uptake sites in brain are very important not only because they contribute to the dopamine removal from the synaptic cleft, but also because they are routes for the entry of neurotoxins and catecholamine precursors into catecholaminergic neurons (Javitch and Snyder, 1984; Chiba et al., 1985). Thus, information about the biochemistry and function of the dopamine transporter is important for understanding the normal physiology of the dopaminergic system and the effects of toxins or drugs that are either taken up by or modulate this transport system.

The binding of dopamine uptake inhibitors to specific sites of the dopamine carrier requires the presence of Na^+ (Kennedy and Hanbauer, 1983; Javitch et al., 1984; Dubocovich and Zahniser, 1985; Janowsky et al., 1986). Na^+-dependence has also been observed for the binding of serotonin (Briley and Langer, 1981) and norepinephrine (Lee et al., 1982; Rehavi et al., 1982) uptake inhibitors. Na^+-dependent cocaine binding sites are decreased by 72% after lesion of dopaminergic nerve terminals with 6-hydroxydopamine, whereas intrastriatal injections of kainic acid that fail to destroy nerve endings do not alter Na^+-dependent cocaine binding sites (Kennedy and Hanbauer, 1983). These data, together with those of Pimoule et al. (1983) showing the lack of cocaine binding sites in corpus striatum of patients with Parkinson's disease, suggest that these bind-

Received August 7, 1990; revised manuscript received November 22, 1990; accepted December 7, 1990.
Address correspondence and reprint requests to Dr. I. Hanbauer at Laboratory of Chemical Pharmacology, NHLBI, National Institutes of Health, Bldg. 10, Rm. 8N-102, Bethesda, MD 20892, U.S.A.

Abbreviations used: KRH, Krebs-Ringer-Henseleit; PBS, phosphate-buffered saline.

ing sites may be closely associated with the dopamine transporter system. In addition, [^{3}H]cocaine binding is inhibited by other high-affinity dopamine uptake blockers, whereas relatively high concentrations of dopamine are required to displace cocaine from its binding sites (Kennedy and Hanbauer, 1983; Reith et al., 1983).

In this article we characterize the binding sites of dopamine uptake blockers in intact, mesencephalic neurons in primary culture and study the time course of expression of cocaine binding sites and specific dopamine uptake sites in these neurons. Our data indicate that cocaine binding sites in primary cultures of ventral mesencephalon derived from 14-day-old rat embryos are expressed simultaneously with the dopamine transporter during development in vitro. Cocaine binding sites in primary cultures of embryonic mesencephali are located in a cytosolic compartment that is not retained in permeabilized cells.

MATERIALS AND METHODS

Ventral mesencephalic cell cultures

Cell cultures were prepared as described by Prochiantz et al. (1979). Because nigral neurons are generated during embryonic days 12–15 (Hanaway et al., 1971; Marchand and Poirier, 1983), female Sprague–Dawley rats (Zivic Miller, PA, U.S.A.), 14 days in gestation, were decapitated, and the embryos were removed and collected in phosphate-buffered saline (PBS), containing 6 mg/ml of glucose. The rostral portion of the ventral mesencephalon of each fetus (3.5 × 3.5 × 1.0 mm) was dissected under sterile conditions, and the tissue was mechanically dissociated in complete culture medium using a fire-polished Pasteur pipette. Cells were centrifuged (100 *g* for 3 min), resuspended in complete medium, and cultured in multiwell plates (16 mm diameter wells; Costar, Cambridge, MA, U.S.A.) previously coated with poly-D-lysine (5 μg/ml, 53,000 MW; Sigma Chemical, St. Louis, MO, U.S.A.). Cells were plated at a density of 30,000–50,000/cm^2 and cultured for 2–12 days at 37°C in an atmosphere of 95% air and 5% CO_2 saturated with H_2O. After 5 days in culture, the cells appeared flattened and usually had two processes emerging from opposite ends of the cell body. Occasionally, cells with three or four processes could also be visualized. A phase-contrast micrograph of mesencephalic cells cultured for 5 days is shown in Fig. 1(1). One of 12 neurons shown in phase contrast is dopaminergic as indicated by uptake of autofluorescent 5,7-dihydroxytryptamine (5 m*M* for 30 min) [Fig. 1(2)]. Fluorescence micrographs were prepared as described by Silva et al. (1988).

Culture medium was composed of a 1:1 mixture of modified minimum essential medium (GIBCO) and nutrient mixture F-12 (GIBCO), supplemented with 15% horse serum (Hyclone), 6 mg/ml of glucose, 2 m*M* glutamine (GIBCO),

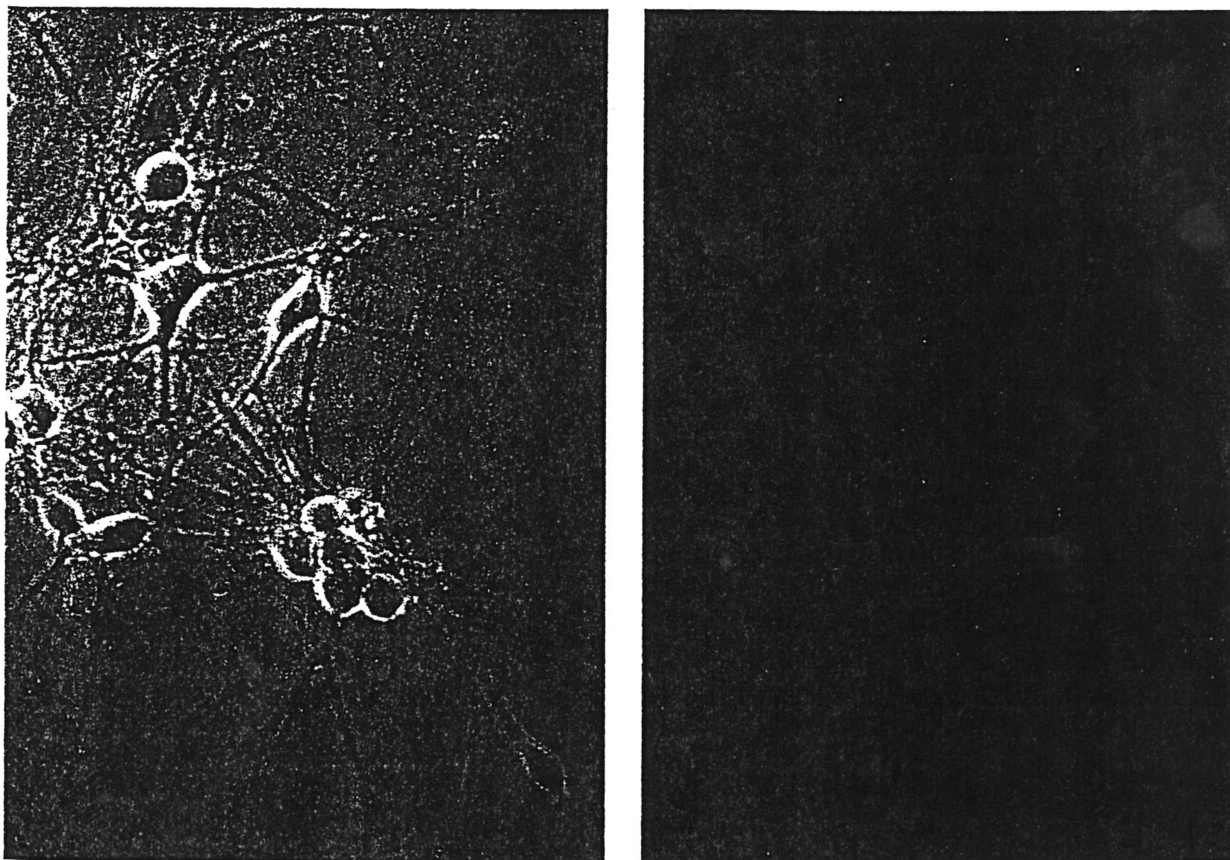

FIG. 1. 1: Phase-contrast micrograph of cells from ventral mesencephalon of 14-day-old rat embryos grown in culture for 5 days. **2:** One of the cells shown in phase contrast is dopaminergic as indicated by the uptake of autofluorescent 5,7-dihydroxytryptamine.

and 0.5 U/ml of penicillin G and 0.5 mg/ml of streptomycin (GIBCO).

[^{3}H]Dopamine uptake

After the cells in each well were washed with 1 ml of Krebs-Ringer-Henseleit (KRH) buffer, they were incubated for 15 min at 37°C in the presence of 5×10^{-8} *M* [^{3}H]dopamine (sp. act. 5–10 Ci/mmol; New England Nuclear) in KRH buffer. Uptake was stopped by removing the incubation solution and rinsing the cells three times with ice-cold KRH buffer. To determine the residual intracellular radioactivity, the cells were lysed and removed from the wells with 0.5 ml of 0.2 *M* NaOH containing 0.2% Triton X-100; the wells were rinsed with an equal volume of 0.2 *M* HCl containing 0.2% Triton X-100, and the combined solutions were added to 10 ml of Ecoscint A (National Diagnostics, Manville, NJ, U.S.A.) to measure the radioactivity with a scintillation spectrometer. The nonspecific dopamine uptake was determined in the presence of 10^{-6} *M* cocaine-HCl (Merck Sharp and Dohme) or 10^{-6} *M* benztropine mesylate (Research Biochemicals, Inc.). Passive diffusion of [^{3}H]dopamine into the cells was determined by incubating the cells at 4°C under the same conditions. The composition of the KRH buffer was as follows: NaCl 136 m*M*, KCl 5 m*M*, $MgSO_4 \cdot 7H_2O$ 0.8 m*M*, $NaHCO_3$ 2.6 m*M*, KH_2PO_4 0.4 m*M*, $Na_2HPO_4 \cdot 7H_2O$ 0.34 m*M*, glucose 5.6 m*M*, HEPES 15 m*M*, $CaCl_2$ 1.3 m*M*, ascorbic acid 0.01%, and pargyline 0.1 m*M*, pH 7.38. Na^+-free KRH buffers were prepared by substituting NaCl with equimolar concentrations of choline chloride or sucrose.

[^{3}H]Cocaine binding in membrane preparations

For measurements of [^{3}H]cocaine binding in membranes of cultured ventral mesencephalic neurons, each culture dish was washed twice with PBS. Then, the cells were scraped off the bottom of the culture dish, dispersed in PBS, and centrifuged for at 20,000 rpm 10 min. The pellet was washed once with 25 m*M* sodium phosphate buffer, pH 7.4, and was then resuspended in the same buffer by sonication using a Polytron (setting 6 for 15 s). The binding of [^{3}H]cocaine (sp. act. 28.5 Ci/mmol; New England Nuclear) (50–1,000 n*M*) in the presence of 50 m*M* NaCl was measured in aliquots of membrane suspension, containing 90–150 μg of protein. Nonspecific binding was determined in the presence of 50 μ*M* cocaine-HCl. The incubation was stopped by rapid filtration through Whatman GF/B filters previously soaked in 25 m*M* sodium phosphate, pH 7.4, containing poly-L-lysine (50 mg/100 ml). The protein content of the membrane suspensions was determined by the method of Lowry et al. (1951).

Specific binding of [^{3}H]cocaine and [^{3}H]mazindol in intact mesencephalic cells

Ventral mesencephalic neurons grown in primary culture for various days were washed twice with 1 ml of KRH buffer and then incubated with KRH buffer containing different concentrations of [^{3}H]cocaine (sp. act. 28.5 Ci/mmol; New England Nuclear) (100–800 n*M*) or [^{3}H]mazindol (sp. act. 30.0 Ci/mmol; New England Nuclear) (1–40 n*M*) for various time periods either at room temperature or at 4°C, respectively. The incubation was terminated by aspirating the incubation solution, followed by three washes of the cells with 0.5 ml of cold KRH buffer to remove the free ligand. Thereafter, the cells were dispersed in 0.5 ml of 0.2 *M* NaOH containing 0.2% Triton X-100, and the wells were washed with an equal volume of 0.2 *M* HCl containing 0.2% Triton X-100. Both solutions were combined and mixed with Ecoscint A, and the amount of radioactivity was determined by scintillation spectrometry. [^{3}H]Cocaine binding occurring at 4 and 37°C was also measured under the same conditions. To determine whether [^{3}H]cocaine may have entered and left the cells by rapid passive diffusion, we washed the wells four times with KRH buffer by incubating each well with ice-cold KRH buffer for 1 min and measured the radioactivity contained in the wash by scintillation spectrometry. The specific binding of the radioligands was calculated as total binding minus nonspecific binding that was obtained in the presence of nonradioactive cocaine (50 μ*M*) or mazindol (2 μ*M*). The average values from saturation isotherms were used to obtain the Scatchard plot. The kinetic parameters were determined by the LIGAND computer program (Munson and Rodbard, 1980).

Permeabilization of cultured mesencephalic neurons

The cell cultures were washed three times with PBS and then preincubated in the presence of streptolysin O (0.15 and 0.20 U/ml; Wellcome Diagnostics, Beckenham Kent, U.K.) for 5 min at 37°C, as described by Howell and Gomperts (1987). The reaction was terminated by aspirating the incubation medium. Permeabilization of cells was verified by brown staining of cells after exposure to ethidium bromide.

Statistical analysis

The significance of differences between experimental results was estimated by using a one-way analysis of variance with post hoc *t* tests.

RESULTS

In an attempt to study whether the expression of the dopamine transporter and binding sites of dopamine uptake inhibitor were linked to the development of neuritic processes, we observed very poor or no specific binding of [^{3}H]cocaine when measured in sonicated and washed, total membrane preparations of mesencephalic neurons cultured for 5 days or even as long as 12 days. Similarly, the specific binding for [^{3}H]mazindol was barely detectable in these types of membrane preparations (data not shown). In fact, the data shown in Fig. 2A demonstrate that in mesencephalic neurons cultured for 12 days, the total and nonspecific binding of [^{3}H]cocaine were not significantly different. This finding was unexpected because cultured mesencephalic neurons possess cocaine-sensitive or mazindol-sensitive [^{3}H]dopamine uptake sites (total [^{3}H]dopamine uptake, 6.4 ± 0.29 pmol/well; [^{3}H]dopamine uptake in the presence of 10^{-6} *M* cocaine, 2.2 ± 0.13 pmol/well; [^{3}H]dopamine uptake in the presence of 10^{-6} *M* mazindol, 1.9 ± 0.20 pmol/well). To elucidate the reason for the apparent discrepancy between these results, we have measured the binding of dopamine uptake blockers in intact cells. The data in Fig. 2B show that specific binding sites for [^{3}H]cocaine are expressed in intact mesencephalic neurons grown 12 days in vitro. The difference between total and nonspecific binding is statistically significant in the presence of 100 and 600 n*M* [^{3}H]cocaine. Specific binding of [^{3}H]cocaine in the presence of 100 or 600 n*M* was about 53% of the total ligand binding.

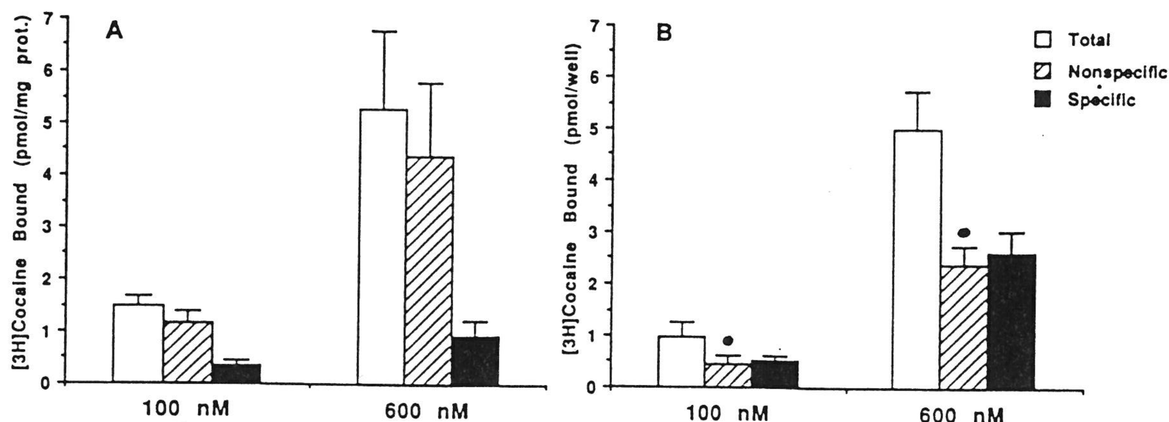

FIG. 2. [^{3}H]Cocaine binding sites in mesencephalic neurons cultured for 12 days. The values represent the mean ± SEM of six measurements performed in the presence of 100 or 600 n*M* [^{3}H]cocaine. Nonspecific binding was determined in the presence of 50 μ*M* cocaine. **A:** Cells cultured for 12 days (6 × 10^5 cells/well) were washed twice with PBS, suspended in 25 m*M* sodium phosphate buffer, pH 7.48 (24°C), and centrifuged at 48,000 *g* for 15 min. The pellet was resuspended in the same buffer by Polytron (setting 6; 15 sec). Aliquots of the membrane suspension containing 100–150 μg of protein were incubated for 30 min at 24°C, as described in Materials and Methods. **B:** Cells cultured for 12 days (6 × 10^5 cells/well; protein content/well = 55–65 μg) were washed twice with PBS and then incubated in KRH buffer containing 100 or 600 n*M* [^{3}H]cocaine in the absence or presence of 50 μ*M* cocaine, as described in Materials and Methods. The black dots indicate statistical significance between total and nonspecific [^{3}H]cocaine binding. $p < 0.05$.

Comparison of the specific binding of [^{3}H]cocaine to intact neurons with that of membranes prepared from these neurons expressed per protein content showed that, under saturating conditions, the density of [^{3}H]cocaine binding sites per milligram of protein is 46-fold higher in intact mesencephalic neurons than in washed and sonicated membranes prepared of cultured mesencephalic neurons. The data in Table 1 show that in intact neurons, the specific binding of [^{3}H]cocaine (100 or 600 n*M*) was similar at 4, 25, or 37°C. Moreover, in the presence of 100 n*M* [^{3}H]cocaine, the specific [^{3}H]cocaine binding was equilibrated after 10 min of incubation at 25°C (Table 2). The results shown in Table 3 rule out the possibility for artifactual binding sites of [^{3}H]cocaine to the surface of the culture dishes. [^{3}H]Cocaine binds only minimally to poly-D-Lysine–coated wells in the absence of mesencephalic cells. The data in Table 3 also show that the binding of [^{3}H]cocaine was not caused by ion trapping of [^{3}H]cocaine after passive diffusion into intact mesencephalic neurons because several consecutive washes of the cells in each well removed free [^{3}H]cocaine but failed to wash out [^{3}H]cocaine that was bound to mesencephalic neurons. The specific binding of [^{3}H]cocaine was fully reversible by adding 10^{-6} *M* cocaine and was inhibited by mazindol (IC_{50} = 5 × 10^{-6} *M*) and benztropine mesylate (IC_{50} = 7 × 10^{-6} *M*), but not by dopamine (5 × 10^{-6} *M*).

Unlike [^{3}H]cocaine binding in striatal membrane preparation of adult rats, that in primary cultures of embryonic mesencephali did not require extracellular Na^+. The results in Table 4 show that [^{3}H]dopamine uptake was impaired when the NaCl of the KRH buffer was replaced by either sucrose or choline chloride. In contrast, [^{3}H]cocaine binding in intact mesencephalic neurons was independent of Na^+ in the KRH buffer and occurred to a similar extent when Na^+ was replaced by equimolar amounts of choline chloride or sucrose.

Saturation isotherms for [^{3}H]cocaine and [^{3}H]-mazindol in intact mesencephalic neurons cultured for 5 days are shown in Fig. 3. Scatchard analysis of

TABLE 1. *Effect of temperature on [^{3}H]cocaine binding in primary cultures of mesencephalic neurons grown for 5 days*

[^{3}H]Cocaine (n*M*)	[^{3}H]Cocaine bound (pmol/well) 4°C	25°C	37°C
200	0.90 ± 0.11	1.23 ± 0.19	0.78 ± 0.26
600	2.97 ± 0.08	3.51 ± 0.53	2.76 ± 0.25

Data are the mean ± SEM from three different experiments performed on cells with dopamine uptake ranging from 2.3 to 3.11 pmol/well (6 × 10^5 cells/well).

TABLE 2. *[^{3}H]Cocaine binding in intact mesencephalic neurons cultured for 5 days*

Incubation time (min)	[^{3}H]Cocaine bound (pmol/well)
10	2.33
30	2.74
60	2.24

Data are the mean of two experiments in triplicate. Each well contained 7 × 10^5 cells. [^{3}H]Cocaine binding was measured in the presence of KRH buffer containing 600 n*M* [^{3}H]cocaine, and nonspecific [^{3}H]cocaine binding was determined in the presence of 20 μ*M* cocaine.

TABLE 3. *Specific binding sites of [^{3}H]cocaine in intact primary cultures of mesencephalic neurons cultured for 5 days*

	[^{3}H]Cocaine (cpm/poly-D-lysine-coated wells)			
	Without cells		600.000 cells/well	
	Total	Nonspecific	Total	Nonspecific
Washes with PBS (n)				
1	9.674	5.352	11.129	13.953
2	778	331	3.240	1.829
3	144	97	1.364	872
4	—	—	897	460
0.2 *M* NaOH + 0.2% Triton X-100	660	610	5.784	2.793

Data are the average of two experiments in triplicate. 100 n*M* [^{3}H]cocaine contained in 0.5 ml of KRH buffer was added to each well and incubated for 15 min at 24°C in the absence or presence of 50 μ*M* cocaine. The reaction was terminated by aspirating the incubation medium. Into each well, 0.5 ml of cold KRH buffer was added and removed after 1 min. This step was repeated three times. The amount of [^{3}H]cocaine contained in the washes was determined by scintillation spectrometry. The residual [^{3}H]cocaine in the cells was extracted with 0.2 *M* NaOH/0.2% Triton X-100.

[^{3}H]mazindol binding indicated the presence of a single class of binding sites (K_D = 20 n*M*; B_{max} = 0.26 pmol/6 × 10^5 cells/well). In contrast, [^{3}H]cocaine binding sites have a fourfold higher K_D value than striatal membranes from adult rats (K_D = 1.3 μ*M*; B_{max} = 7.2 pmol/6 × 10^5 cells/well). Because the present data provided evidence that [^{3}H]cocaine and [^{3}H]mazindol binding sites are present in intact cells but barely detectable in washed membrane preparations of cells, it can be surmised that [^{3}H]cocaine binding may either occur in a cytosolic compartment that is removed during the washing procedure or may require intact neuronal membranes. To analyze these possibilities, we studied [^{3}H]cocaine binding in permeabilized cells. The data in Table 5 show that permeabilization of cultured mesencephalic neurons by incubation with 0.15 or 0.20 U/ml of streptolysin O reduces [^{3}H]cocaine binding to 41 and 6% of control, respectively. In contrast, the storage of [^{3}H]dopamine in cultured mesencephalic neurons was not markedly altered when cells were permeabilized with streptolysin O after [^{3}H]dopamine was taken up. The data in Table 5 show that [^{3}H]dopamine remained stored in the cells and failed to leak out into the incubation medium. In contrast, if cultured cells were permeabilized with streptolysin O before uptake was measured, the [^{3}H]dopamine uptake was reduced to 67% of control after treatment with 0.20 U/ml of streptolysin O by using the same incubation conditions as described for [^{3}H]dopamine uptake. At the present time, we do not understand whether this decrease is due to a direct effect of streptolysin O on storage sites or to leakage of soluble cytosolic proteins that are operative in dopamine transport.

Because binding sites for dopamine uptake blockers are considered to be closely associated with the dopamine transporter, we investigated the developmental expression of [^{3}H]dopamine uptake and [^{3}H]cocaine binding sites in primary cultures of neurons. The data in Fig. 4 demonstrate that the dopamine uptake and cocaine recognition sites are expressed simultaneously and their density increases proportionally in cultured mesencephalic neurons.

TABLE 4. *Effect of Na^+-free buffers on [^{3}H]dopamine uptake and [^{3}H]cocaine binding sites in intact mesencephalic neurons cultured for 5 days*

	[^{3}H]Dopamine uptake (pmol/well)	[^{3}H]Cocaine bound (n*M*) 100	[^{3}H]Cocaine bound (n*M*) 600
KRH buffer	3.1 ± 0.11 (n = 7)	0.99 ± 0.097 (n = 7)	3.8 ± 0.43 (n = 7)
Choline[a]	0.42 ± 0.055[b] (n = 4)	0.75 ± 0.10 (n = 4)	4.8 ± 0.80 (n = 4)
Sucrose[a]	0.39 ± 0.071[b] (n = 3)	0.87 ± 0.25 (n = 3)	3.3 ± 0.80 (n = 3)

Data are the mean ± SEM (6 × 10^5 cells were plated per well).

[a] NaCl (136 m*M*) contained in KRH buffer was substituted by equimolar amounts of choline chloride or sucrose.

[b] $p < 0.01$, when compared with KRH buffer-incubated cells.

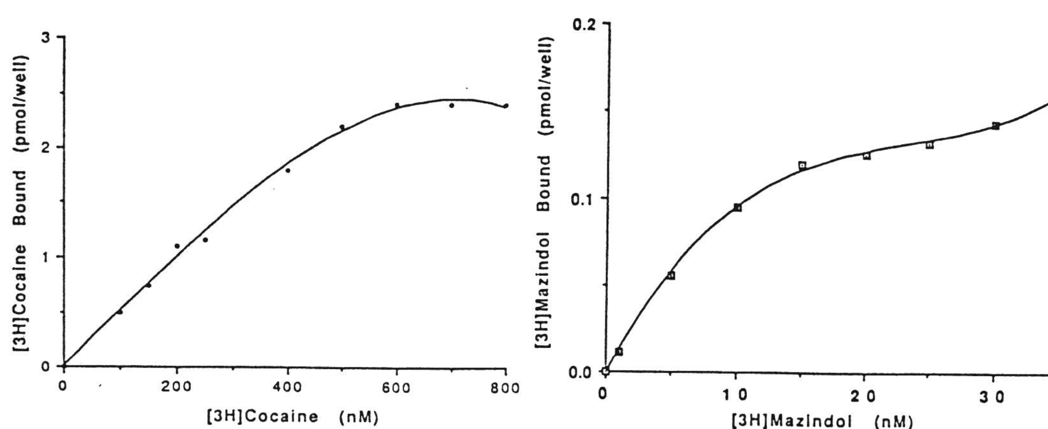

FIG. 3. Saturation isotherms for [³H]cocaine and [³H]mazindol in intact mesencephalic neurons cultured for 5 days. Each well contained 6×10^5 cells. Each data point is the average of two experiments in triplicate. The kinetic parameters for the radioligand binding were determined by the LIGAND computer program (Munson and Rodbard, 1980).

DISCUSSION

Although the inhibition of the dopamine transporter by central stimulants chemically unrelated to amphetamine has been extensively studied, the site of action and the molecular mechanisms that operate in this inhibition are still unknown. The results presented here demonstrate that in primary cultures of ventral mesencephalon from rat embryos, dopamine uptake and binding sites for dopamine uptake blockers are expressed simultaneously and increase proportionally during development in vitro. Unlike [³H]cocaine binding sites in corpus striatum of adult rats, where they are unambiguously located in synaptosomal membranes, those in primary cultures of ventral mesencephalon are scarce in plasma membranes but appear to be located mainly in a cytosolic compartment. Previous work demonstrated that [³H]cocaine or [³H]-mazindol binding to washed striatal membrane preparations of adult rat is a saturable and Na^+-dependent process (Kennedy and Hanbauer, 1983; Javitch et al., 1984). Surprisingly, in washed and disrupted membranes prepared from mesencephalic neurons cultured for 5 or even as long as 12 days, specific binding sites for [³H]cocaine or [³H]mazindol were barely detectable, although intact neuronal cultures expressed dopamine uptake that could be inhibited by cocaine, mazindol, or benztropine mesylate. One explanation for this phenomenon could be that the rate of dissociation of the ligand-receptor complex is faster in cultured mesencephalic neurons derived from embryos than in striatal tissue of adult rats. Because [³H]cocaine had very low specific binding in disrupted membranes of cultured neurons, it was not possible to determine the rate of dissociation of the radioligand-receptor complex. The attempt to terminate the binding assay by centrifugation instead of filtration technique, however, resulted also in low specific [³H]cocaine binding (data not shown).

In contrast, [³H]cocaine and [³H]mazindol binding sites were detectable in primary cultures of intact mesencephalic neurons using the same incubation buffer as described for [³H]dopamine uptake studies. Under these conditions, neither [³H]cocaine nor [³H]mazindol bound to poly-D-lysine–coated culture dishes used for primary cultures, eliminating the possibility of artifactual radioligand binding. Moreover, the present experiments ruled out that [³H]cocaine binding in intact cells may be caused by ion trapping after passive diffusion because repeated washes of the cells with ice-cold buffer did not remove specifically bound [³H]cocaine. Internalization of [³H]cocaine by endocytosis, which is known to be a temperature-dependent

TABLE 5. *[³H]Cocaine binding and [³H]dopamine storage in intact and permeabilized primary cultures of mesencephalic neurons*

Streptolysin O (U/ml)	[³H]Cocaine bound (pmol/well)[a]	[³H]Dopamine (pmol/well/15 min)[b] Incubation buffer	Cells
None	0.44 ± 0.14	0.43 ± 0.022	1.9 ± 0.060
0.15	0.18 ± 0.035[c]	0.57 ± 0.045	2.0 ± 0.065
0.20	0.028 ± 0.021[c]	0.50 ± 0.027	1.6 ± 0.087

Data are the mean ± SEM (n = 5).

[a] The wells were preincubated at 37°C for 5 min with 0.5 ml of PBS containing 33.3 mM glucose in the absence or presence of 0.15 or 0.20 U/ml of streptolysin O. [³H]Cocaine binding was determined in PBS containing 6 mg/ml of glucose and 100 nM [³H]cocaine. Nonspecific [³H]cocaine binding was determined in the presence of 20 μM cocaine.

[b] The wells were incubated at 37°C for 15 min with KRH buffer containing 10 μM pargyline and 50 nM [³H]dopamine. Nonspecific uptake of [³H]dopamine was measured in the presence of 10^{-6} M cocaine. The wells were washed three times with ice-cold KRH buffer and then incubated at 37°C for 5 min with 0.5 ml of PBS containing 6 mg/ml of glucose in the presence of 0.15 or 0.20 U/ml of streptolysin O. The amount of [³H]dopamine released into the incubation buffer and of that retained in the cells was determined.

[c] $p < 0.001$.

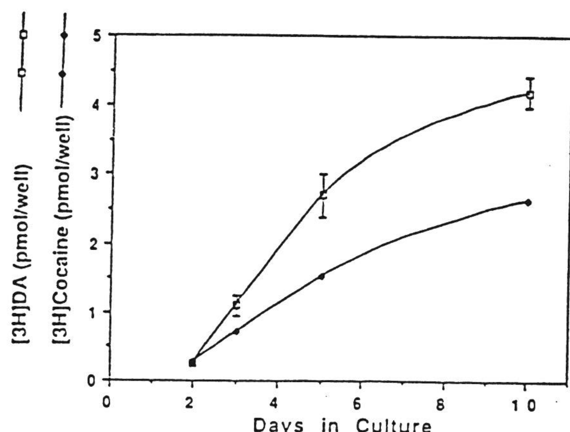

FIG. 4. In vitro development of [^{3}H]dopamine uptake sites and [^{3}H]cocaine binding sites in primary cultures of mesencephalic neurons. Each well contained 6 × 10^5 cells. The data points are the mean ± SEM (n = 6). [^{3}H]Dopamine uptake/well was measured at 37°C for 15 min, as described in Materials and Methods. The specific binding of [^{3}H]cocaine/well was measured at 24°C for 10 min, as described in Materials and Methods.

process, can also be excluded because specific binding of [^{3}H]cocaine to intact mesencephalic neurons occurred to a similar extent at 4, 24, or 37°C.

Instead, the present results suggest that in intact cultured neurons, [^{3}H]cocaine may not bind to recognition sites that are anchored in the outer neuronal membrane, but may rather bind to a cytosolic soluble protein or intracellular organelles. Recent work by Zaczek et al. (1990) demonstrated that amphetamine-like compounds diffuse into crude striatal synaptosomes at 4°C, where they are sequestered by an intrasynaptosomal component. This mechanism was deduced from findings showing that sonication or digitonin treatment of striatal synaptosomes reduced the incorporation of amphetamine-like substances. Similarly, our results show that [^{3}H]cocaine binding sites were not retained in streptolysin O-permeabilized cells, suggesting that in cultured mesencephalic neurons, unlike in adult neurons, specific binding of dopamine uptake blockers may occur at the level of a soluble cytosolic protein. This possibility is further supported by the lack of finding of [^{3}H]cocaine binding sites in membrane preparations of cultured neurons. Because this preparation included sonication and wash steps, soluble proteins carrying putative binding sites for cocaine may have been washed out. Our studies also showed that in mesencephalic neurons, [^{3}H]dopamine storage sites, which may include small vesicles, and/or smooth endoplasmic reticulum (Geffen et al., 1976; Mercer et al. 1979) are retained within the cells during permeabilization with streptolysin O and fail to leak through the perforated membranes into the incubation medium. In contrast, we have consistently observed a reduction in [^{3}H]dopamine uptake if uptake is measured in already permeabilized cells, suggesting that the cytosolic soluble protein that leaked through the pores may play a functional role in the [^{3}H]dopamine uptake process. Our results also indicate that the pharmacological properties of [^{3}H]cocaine binding sites in intact cultured mesencephalic neurons are similar to those in sonicated membranes of adult rats. The binding of [^{3}H]cocaine to intact neurons was inhibited by benztropine mesylate, cocaine, or mazindol, with IC_{50} values similar to those reported for striatal membranes of adult rats (Kennedy and Hanbauer, 1983).

Interestingly, in intact primary cultures of mesencephalic neurons, we observed that binding of [^{3}H]cocaine remained unchanged when Na^+ was stoichiometrically substituted by choline chloride or sucrose. This is in contrast to data obtained in striatal membranes of adult rats showing that replacement of NaCl by choline chloride in the incubation buffer inhibited [^{3}H]cocaine binding (M. Grilli and I. Hanbauer, unpublished data). The mechanism whereby Na^+ facilitates cocaine binding to dopaminergic nerve terminals is not clearly understood. Reports on other radioligands attributed Na^+-induced increase of radioligand binding to conformational alteration of the receptors (Pasternak et al., 1975) or stabilization of the ligand-receptor complex (Usdin et al., 1980). Some discrepancies exist, however, between the Na^+ requirements for binding of dopamine uptake inhibitor and dopamine uptake. Benmansour et al. (1987) suggested that when sodium is the only cation present in the incubation medium, the binding of [^{3}H]GBR 12783, a very potent and selective inhibitor of the dopamine uptake, is sodium dependent within a low range of ion concentrations, a profile that is very different from that observed in buffers containing additional ions such as K^+, Ca^{2+}, Mg^+, $Tris^+$, and $choline^+$.

Our present data suggest that [^{3}H]cocaine binding requires Na^+ when the binding sites are anchored within the neuronal membrane, but appears to be Na^+-independent when the binding sites are associated with a cytosolic compartment. An internalization of [^{3}H]cocaine binding sites into neuronal membranes may occur during ontogenesis. This hypothesis is supported by preliminary evidence indicating that [^{3}H]cocaine binding was barely detectable in striatal membrane preparations of 19-day-old embryos or of newly born rats, but was well expressed in 5-day-old rats (M. Grilli, U. di Porzio, and I. Hanbauer, unpublished data).

Acknowledgment: The authors thank Dr. A. P. Mariani for preparing the phase contrast and fluorescence micrographs. Dr. M. Grilli was supported in part by a research fellowship from FIDIA, Abano Terme, Italy.

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