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MOLECULAR MECHANISMS INVOLVED IN TRANSPORT AND RELEASE OF DOPAMINE IN PRIMARY CULTURES OF MESENCEPHALIC NEURONS

I. HANBAUER and M. GRILLI*

Laboratory of Chemical Pharmacology, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.

Some 40 years ago, biochemical and electrophysiological studies led to the quantal theory of synaptic transmission (Del Castillo and Katz, 1954), and to the discovery showing calcium to be essential in triggering neurotransmitter release (Harvey and MacIntosh, 1940; Del Castillo and Stark, 1952). Later it was observed that neurotransmitters were released from nerve endings by exocytosis of synaptic vesicles (Del Castillo and Katz, 1956). By using the quick-freezing tecnique, Torri-Tarelli and coworkers (1985) demonstrated that fusion of vesicles with the plasma membrane and neurotransmitter release occurred at the same time. Increasing knowledge on the biochemical composition of storage vesicles led to a better understanding of the interaction of vesicles with microtubules and actin-based cytoskeleton (Hirowaka and Heuser, 1982) that appears to be operative in triggering exocytosis.

In contrast, uptake of neurotransmitter by storage vesicles involves transmitter-specific transport proteins that function as specific ATP-driven proteins pumps (Johnson, 1988). Creation of an electrochemical proton-gradient across vesicle membranes mediates the transport of small molecules, such as catecholamines, serotonin, acetylcoline, or glutamate into the vesicles where they are then packaged. The uptake of catecholamines and serotonin can be inhibited by mechanisms including direct inactivation of [^{3}H]ATPase, and perturbation of pH and proton-gradients. In addition, reserpine, guanethidine, and nonamphetamine-like central stimulants, including cocaine, mazindol, or nomifensine (Iversen, 1974) are potent uptake inhibitors. Their action on the amine transporter, however, is poorly understood.

This paper summarizes work carried out in this laboratory on the regulation of dopamine transport and release in primary cultures of mesencephalic neurons. In cultures prepared from the ventral, tegmental mesencephalon of 14 day old rat embryos, 3% to 5% of the cells are dopaminergic. Dopamine uptake in mesencephalic cell cultures is measurable as soon as neurites develop (Prochiantz *et al.*, 1979; Grilli *et al.*, 1991). Fluorescence microscopic studies using autofluorescent 5,7-dihydroxytryptamine for labeling dopaminergic neurons showed that the majority of dopaminergic neurons are bipolar, but some of the neurons have three or four fibers (de Erausquin and Hanbauer, unpublished). It is still unclear whether neurites of multipolar dopaminergic neurons differentiate into one axon and several dendrites in primary cultures as presumably occurs during neuronal development *in situ* (Sargent, 1989). This consideration may be of interest, because storage sites for neurotransmitters have been shown to differ between axons and dendrites. Axonal neurotransmitter storage occurs in synaptic vesicles, while dendritic storage involves the endoplasmic reticulum (Mercer *et al.*, 1979)

DOPAMINE TRANSPORTER IN CULTURED MESENCEPHALIC NEURONS

Previous studies on the inhibition of dopamine uptake in striatal dopaminergic axons by central stimulants showed that these compounds specifically bind to sites located in dopaminergic nerve terminals (Kennedy and Hanbauer, 1983; Javitch *et al.*, 1984; Dubocovich and Zahniser, 1985; Ritz *et al.*, 1990), and that the binding to striatal membrane preparations was a Na^+-requiring process. It was observed that striatal membranes contain high and low-affinity binding sites for [^{3}H]cocaine and its analogs (Calligaro and Eldefrawi, 1988; Ritz *et al.*, 1990) suggesting that these compounds may have multiple binding domains. Although central stimulants are potent blockers of

* Present address: Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, U.S.A.

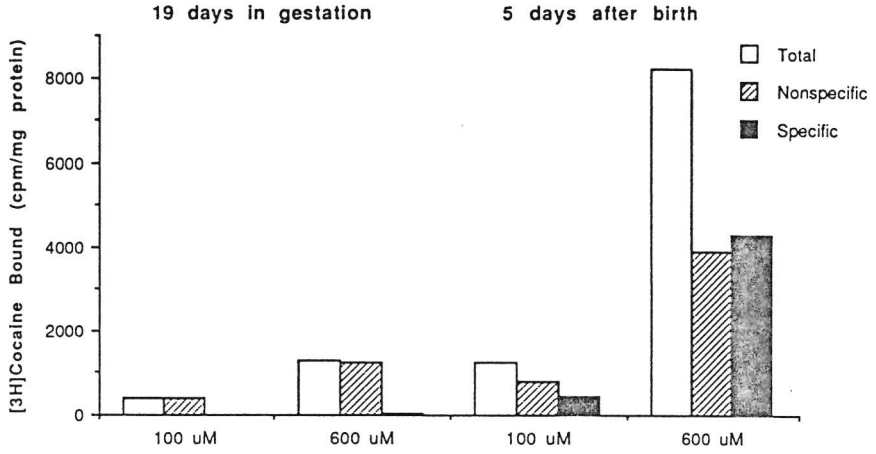

Fig. 1. Specific binding of [^{3}H]cocaine in striatal membrane preparations of 19 day old embryos and rats five days after birth. The bar graph represents the average of two experiments carried out in triplicate. Total binding of [^{3}H]cocaine washed and sonicated striatal membrane was measured in the presence of 25 mM sodium phosphate buffer at pH 7.48, 50 mM NaCl, and 100 nM or 600 nM [^{3}H]cocaine. Nonspecific binding of [^{3}H]cocaine was measured in the presence of 20 μM cocaine.

[^{3}H]dopamine uptake in cultured mesencephalic neurons, we failed to detect specific binding sites of [^{3}H]cocaine or [^{3}H]mazindol in sonicated and washed membranes prepared from cultured mesencephalic neurons (Hanbauer *et al.*, 1990). In contrast, both radioligands interacted with specific binding sites when intact mesencephalic neurons were used. Moreover, in intact cultured neurons the specific binding of [^{3}H]cocaine was fully reversible by adding 10^{-6} M cocaine and was inhibited by other specific uptake dopanine blockers, such as mazindol ($IC_{50} = 5\mu M$) or benztropin mesylate ($IC_{50} = 7\ \mu M$). The specific binding of [^{3}H]cocaine achieved equilibrium within 10 min. of incubation and occurred at a similar rate at 4°C, 24°C and 37°C. The lack of a temperature effect on [^{3}H]cocaine binding in cultured mesencephalic neurons demonstrates that [^{3}H]cocaine is transported across neuronal membranes by passive diffusion and not by an active transport process. It appears that within mesencephalic neurons cocaine binds to a cytostolic binding site. This inference is supported by experiments showing that [^{3}H]cocaine was not removed by washing the cells, but leaked out from cells that were permeabilized with streptolysin-O (Hanbauer *et al.*, 1990; Grilli *et al.*, 1991). Interestingly, we also observed an absence of [^{3}H] cocaine binding sites in membranes prepared from corpus striatum of 19 day old embryos (Fig.1), although [^{3}H]dopamine uptake was already operative in this tissue (U. Di Porzio, personal communication). We were able to measure specific binding sites of cocaine in sonicated and washed striatal membranes of rats as early as 5 days after birth (Fig. 1). In contrast to the mechanism of [^{3}H]cocaine binding to striatal membranes of adult rats, [^{3}H]cocaine binding to cultured mesencephalic neurons was Na^+-independent (Fig.2). The data in Fig. 2 (bottom panel) show that [^{3}H]cocaine binding in cultured mesencephalic neurons was similar in normal Krebs-Ringer-Henseleit buffer (KRH). and in KRH with Na^+ substituted by equimolar amounts of either choline chloride or sucrose. Absence of Na^+ in the KRH inhibited the uptake of [^{3}H]dopamine in cultured mesencephalic neurons (Fig. 2, top panel). These results suggest that in fetal and neonatal corpus striatum [^{3}H]cocaine may not bind to neuronal membranes but to a cytosolic component within the axons and may be expressed in membranes during postnatal development. It is tempting to speculate that in primary cultures of embryonic mesencephalon the expression of [^{3}H]cocaine binding sites in neuronal membranes may depend on trophic factors that are missing in the presently used culture media.

CONTROL OF DOPAMINE RELEASE IN CULTURED MESENCEPHALIC NEURONS

Voltage-sensitive Ca^{++} channels possess different biophysical and pharmacological properties in cell bodies and nerve endings (Sanna *et al.*, 1986; Miller, 1987; Hirning, *et al.*, 1988; Llinas *et al.*, 1989). It has been suggested that the calcium channels in nerve

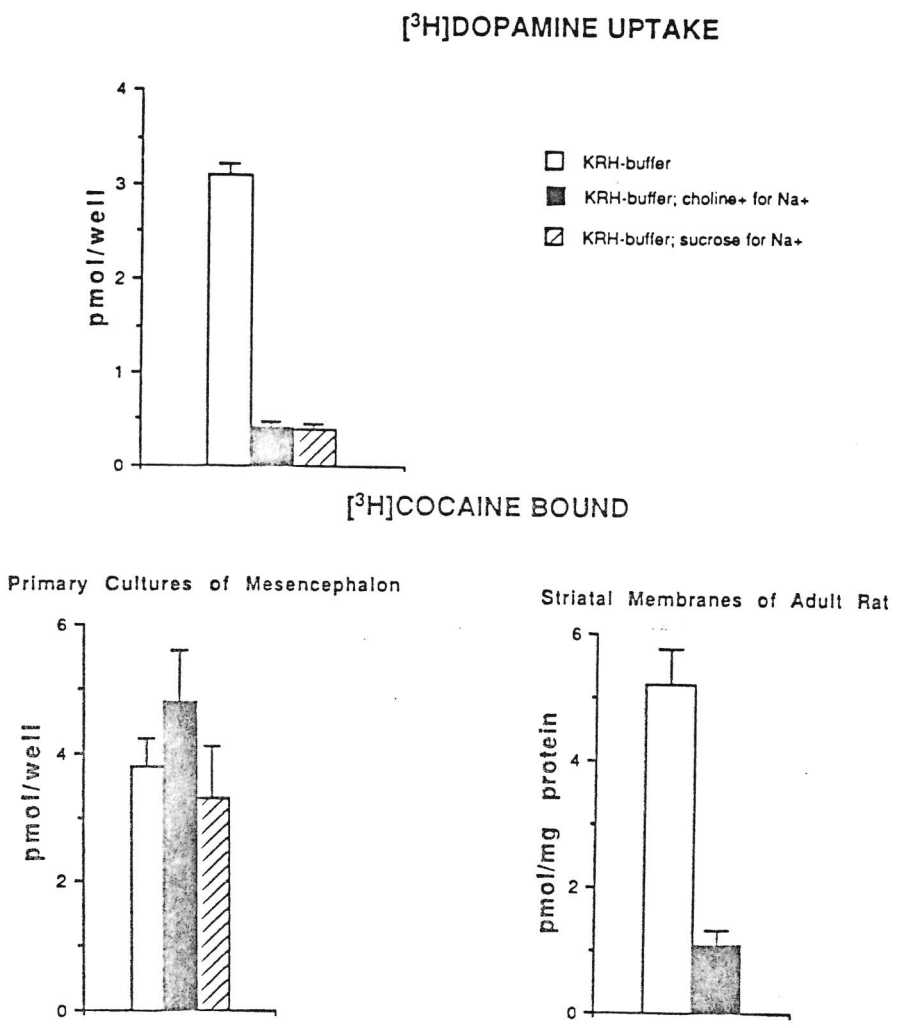

Fig. 2. Differences in Na^+-requirement for [^{3}H]dopamine uptake and [^{3}H]cocaine binding. [^{3}H]Dopamine uptake and [^{3}H]cocaine binding were measured in normal Krebs-Ringer-Henseleit (KRH) buffer, or Na^+ substituted with equimolar amounts of choline chloride, or sucrose. The values are the mean ± SEM of five measurements, and are expressed as pmol [^{3}H]dopamine/well, or pmol [^{3}H]cocaine bound/well. In each well 6×10^5 cells were plated and grown in vitro for five days.

terminals are predominantly the N-type, while L- and T-type calcium channels are found mainly in perikarya. Work carried out in this laboratory revealed that [^{3}H] dopamine release elicited by different types of depolarizing agents from cultured mesencephalic neurons usually required extracellular Ca^{++} (Grilli *et al.*, 1989).

The K^+-evoked release of [^{3}H]dopamine was inhibited by the N-type channel blockers, ω-conotoxin and neomycin, in a dose dependent manner (Grilli *et al.*, 1989). Although ω-conotoxin also blocks Ca^{++} currents through L-type channels, the specific L-type channel blocker, nifedipine, had no effect (Table 1).

Moreover, the T-type channel blocker, amiloride, also failed to inhibit [^{3}H]dopamine release (Table 1). The insensivity to nifedipine and to amiloride, but a strong sensitivity to ω-conotoxin can be regarded as a pharmacological criterion for the sole involvement of N-type Ca^{++} channels in [^{3}H]dopamine release from dopaminergic nerve endings. Studies on Ca^{++} flux in fura–2 loaded neuron provided evidence that nifedipine attenuated the K^+-evoked rise in the intracellular Ca^{++} in the soma of dopaminergic neurons in these cultures (de Erausquin and Hanbauer, unpub-

Table 1. The effect of Ca^{++} channel blockers on K^+- and veratridine-evoked [^{3}H]dopamine (DA) release in primary cultures of mesencephalic neurons

Ca^{++} channel inhibitor	Channel type	%Increase over basal [^{3}H]DA release	
		20 mM K^+	2μM Veratridine
None		87	204
0.5 μM Nifedipine	L	86	198
1 μM ω-Conotoxin	N, L	19	209
500 μM Neomycin	N	12	225
100 μM Amiloride	T	111	198

Each value is the mean of two determinations in triplicate and represents the percentage of [^{3}H]DA release over basal level. The Ca^{++} channel blockers were added during a 5 min. preincubation period and were also present during 5 min. incubation with depolarizing agents.

lished results) indicating that nifedipine interacts with receptor sites on the L-type Ca^{++} channel. Based on these findings, however, one may surmise that Ca^{++} transported into the neuron by different types of voltage-dependent Ca^{++} channels may be distributed into different functional pools. On the other hand, blockade of L-type channels by nifedipine may prevent Ca^{++} entry into the soma but not nerve endings, while ω-conotoxin blocks Ca^{++} entry into both neuronal sites. In contrast, the veratridine-evoked release of [^{3}H]dopamine was not inhibited by N-, L-, or T-type Ca^{++} channel blockers (Table 1), but was completely blocked by 0.1 μM tetrodotoxin, and required extracellular Ca^{++}. Veratridine increases the intracellular Ca^{++} content in dopaminergic neurons present in primary cultures of mesencephalic neurons as determined by fura–2 single cell imaging (de Erausquin and Hanbauer, unpublished results). The increased entry of Ca^{++} apparently involving a Na^+/Ca^{++} exchange is mediated by a voltage-dependent Na^+ channel. These data suggest that the increased Ca^{++} influx subserving the release of neurotransmitters can be triggered by various types of ion channels that are activated under membrane depolarizing conditions. The present data establish that the K^+-evoked [^{3}H]dopamine release from cultured mesencephalic neurons is mediated by the N-type voltage-sensitive Ca^{++} channel. At present time we cannot explain why nifedipine fails to block the K^+-evoked [^{3}H]dopamine release, although it attenuated the increase of Ca^{++} influx in dopaminergic perikarya. Ongoing studies using fura–2 single cell digital imaging are aimed at understanding the effect of different types of Ca^{++} channel blockers on Ca^{++} fluxes in perikarya and nerve fibres of cultured mesencephalic neurons.

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