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CHARACTERIZATION OF DOPAMINE BINDING SITES IN STANDARD PREPARATIONS OF BRAIN SYNAPTIC MEMBRANES

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Abstract—Brain synaptic membranes, prepared according to DeRobertis and to Whittaker, were compared morphologically and biochemically. Electron microscopy revealed that DeRobertis preparations were heavily contaminated with synaptosomes, mitochondria, storage vesicles, and a variety of extraneous membrane structures; Whittaker preparations appeared to consist of synaptic membranes, with a relatively small number of presynaptic storage vesicles. Substantial dopamine β-hydroxylase, monoamine oxidase, and catecholamine-*O*-methyl transferase activities were present in DeRobertis preparations; Whittaker membrane preparations contained low-to-undetectable activities of these enzymes. Although dopamine bound to membranes derived from both brain cortex and corpus striatum, only membranes from the latter area contained dopamine-sensitive adenylate cyclase. Binding of dopamine to Whittaker striatal synaptic membrane preparations was relatively rapid, saturable, partially reversible, and did not chemically alter the ligand. A variety of dopamine acceptors appeared to be present in this preparation since the ED_{50} (from Klotz plots) of dopamine binding was 25 times that for activation of adenylate cyclase; Scatchard plots revealed both high and low affinity binding sites for dopamine; and, binding and adenylate cyclase activation studies with dopamine, carried out in the presence of fluphenazine, cocaine, pargyline, and reserpine, reveal that even the more-pure Whittaker preparations of striatal synaptic membranes contain at least three major dopamine-binding components: postsynaptic (and perhaps presynaptic as well) membrane receptors linked to adenylate cyclase, a presynaptic synaptosomal reuptake receptor, and storage vesicle sequestration.

It has been recognized for some time that crude homogenates and synaptosomal fractions, prepared according to DeRobertis *et al.* [1] and to Whittaker *et al.* [2], contain dopamine stimulatable adenylate cyclase activities (Krueger [3]; Karobeth and Leitich [4]; Kebabian *et al.* [5]; Mishra *et al.* [6]; Von Hungen and Roberts [7]). However, binding of dopamine to its specific receptors or acceptors in these preparations has not been characterized in detail; nor has binding of dopamine been correlated kinetically with activation of adenylate cyclase. Furthermore, since the aforementioned tissue preparations are crude, it is quite likely that dopamine also interacts with structures other than receptors linked to adenylate cyclase; purified synaptic membranes have not been studied in this regard.

In this report, we describe the biochemical characterization of the interaction of dopamine with several components present in preparations of brain corpus striatum synaptic membranes purified according to Whittaker *et al.* [2] as modified by Hoss and Abood [8]. Comparisons are made with results obtained using membranes prepared according to DeRobertis *et al.* [1].

MATERIALS AND METHODS

DeRobertis synaptic membrane preparation. In early studies, male Sprague–Dawley rats (Flow Laboratories, Dublin, VA) were rapidly decapitated, the cortex dissected out on ice, and the tissue homogenized as a 20% (w/v) suspension in 0.32 M sucrose (adjusted to pH 7) using a loose-fitting Potter–Elvehjem homogenizer. Synaptosomes (M_1 pellet as described by DeRobertis *et al.* [1]) were prepared from this suspension; these were quick frozen and stored overnight in liquid nitrogen. The following day the thawed synaptosomes were homogenized in ice-cold, glass-distilled water using a tight-fitting Dounce homogenizer, followed by agitation of the suspension for 20 min at 4°. The disrupted nerve terminals were collected by centrifugation, suspended in 0.32 M sucrose, layered on a discontinuous sucrose gradient (0.8, 0.9, 1.0, 1.2 M) and centrifuged at 50,000 *g* for 2 hr at 4° in a Beckman L2-65B centrifuge with a SW 27 rotor. Synaptic membranes were isolated by centrifugation from the 0.9–1.0 M and 1.0–1.2 M layers, suspended in 0.32 M sucrose, and stored as 100 μl aliquots in liquid nitrogen.

Whittaker synaptic membrane preparations. Cortex or striatum, dissected from male Sprague–Dawley rats, and corpus striatum dissected from calf brains, were immediately homogenized, and synaptosomes (0.8–1.2 M intergradient layer) were prepared and isolated as described by Whittaker *et al.* [2] and Hoss and Abood [8]. The collected synaptosomes were stored in liquid nitrogen overnight. The following day

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the frozen synaptosomes were homogenized in glass-distilled water at room temperature using a tight-fitting Dounce homogenizer. The homogenate was then layered on a discontinuous sucrose gradient (0.6, 0.8, 1.0, 1.2 M) and centrifuged with a SW 27 rotor. Synaptic membranes were isolated by centrifugation from the 0.6–0.8 M and the 0.8–1.0 M layers interfaces; these were suspended in 0.32 M sucrose and stored as 100 μl aliquots in liquid nitrogen.

Binding assays. For the standard binding assay, incubation mixtures prepared in duplicate in test tubes at 4° contained 0.1 μM [1-^{3}H]dopamine (8.4 Ci/m-mole; New England Nuclear Co), 0.1 mM dithiothreitol, and 50 mM Tris–HCl (pH 7.8) or 50 mM glycylglycine buffer (pH 8.0) in a final volume of 150 μl. Fluphenazine hydrochloride (Schering Corp.), pargyline hydrochloride (gift from Dr. John Tallman, NIH, Bethesda, MD), cocaine hydrochloride (gift from Dr. Donald Thompson, Georgetown University, Washington, DC), and reserpine (free base, Nutritional Biochemicals) were further additions in studies utilizing inhibitors of dopamine binding or metabolism. Reactions were started by the addition of synaptic membranes (5 to 60 μg protein). Incubations, which were performed for two hr at 37° in a shaker bath under steady-state conditions (cf., Fig. 3), were ended by the addition of 5 ml of ice-cold buffer (as above), immediately followed by the rapid isolation of synaptic membranes by filtration through Millipore filter discs (HAWP-25 mm dia., 0.45 μm pores) under reduced pressure in a Millipore filtration manifold. Filters were prewashed with 0.25 M KCl solution to minimize non-specific adsorption of ligands. Filter discs were washed with cold buffer and then air- or oven-dried. Filters were dissolved in 10 ml of liquid scintillation solution 0.4% PPO–0.1% POPOP in toluene–Triton X-100 (9:8) for quantifiaction of radioactivity in a Nuclear-Chicago Mark II spectrometer. Under these conditions, about 3 per cent of the added dopamine was specifically bound. Non-specific binding of [1-^{3}H]dopamine was defined as the amount remaining bound to membranes in the presence of a large (1000-fold) excess of unlabeled dopamine (obtained as dopamine hydrochloride from Nutritional Biochemicals). No degradation of receptors appeared to occur under the conditions of this binding assay, since purified (but not crude) synaptic membranes could be preincubated for as long as 4 hr with no diminution in the capacity to bind dopamine specifically (G. Bers and M. Blecher, unpublished).

Chromatography of [1-^{3}H]*dopamine.* Descending chromatography, using *n*-butanol–acetic acid–water (25:4:10), a solvent system known to separate dopamine from its metabolic and oxidation products (Karobeth and Leitich [4]; Koretz and Marinetti [9]; Mattock [10]; McGeer and Clark [11]; Roberts and Broadley [12]), were utilized to identify any ^{3}H-ligand which was bound to synaptic membranes. Bound ^{3}H-ligand was removed from membranes by extraction with 1 N HCl for 30 min at 37°, and chromatographed on Whatman No. 1 paper (in an atmosphere of nitrogen) alongside catecholamine standards. After 16–20 hr, the developed chromatogram was air-dried; radioactive spots were detected and quantified by a Brinkman–Berthold two-dimensional radioscanner.

Adenylate cyclase assay. Adenylate cyclase activity of synaptic membrane preparations (5 to 60 μg protein) was assayed under conditions of zero-order kinetics by the Krishna method as modified by Rodbell *et al.* [13] and Birnbaumer and Yang [14]. Briefly, synaptic membranes were incubated ror 15 min at 37° in a 50 μl incubation mixture containing [α-^{32}P]-ATP (35 μCi), 0.4 mM ATP, 1.0 mM cAMP, 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 10 mM $MgCl_2$, 1 mM EDTA, in 30 mM Tris–HCl buffer (pH 7.6). Reactions were ended by the addition of 100 μl of a stopping reagent (12.5 mM cAMP, 40 mM Na_2ATP, 80 nCi [^{3}H]cAMP, and 10 mg/ml sodium lauryl sulfate) and placing tubes in a boiling water bath for 2.5 min. ATP and cAMP were then separated by >99% on a short column (1 ml) of Dowex-50 (H^+) (100–200 mesh) resin, followed by treatment of the eluate with equimolar amounts of $Ba(OH)_2$ and $ZnSO_4$ to achieve complete separation of the two nucleotides [13, 14]. The amounts of [^{32}P]cAMP produced are quantified by dual-label counting in a liquid scintillating spectrometer, with the amount of [^{3}H]cAMP present being used to correct for losses of product during the work-up procedure.

Assays for enzymes metabolizing dopamine. Catechol-*O*-methyl transferase (COMT) was assayed as described by Axelrod [15]. The transfer of label from [*methyl*-^{14}C]-*S*-adenosyl methionine (44 mCi/m-mole; ICN Pharmaceuticals) to epinephrine forming [^{14}C]metanephrine was quantified as a measure of COMT activity.

Monoamide oxidase (MAO) was assayed as described by Axelrod [15]. The conversion of [^{14}C]tryptamine (53 mCi/m-mole; New England Nuclear, Co), to [^{14}C]indoleacetic acid was used as a measure of enzyme activity.

Dopamine β-hydroxylase (DBH) activity was determined by Dr. John Tallman (NIH) according to Molinoff *et al.* [16]. This activity was equal to the amount of label transferred from [*methyl*-^{3}H]-*S*-adenosylmethionine to the 4-hydroxyphenethylamine formed from tryamine by the enzyme.

Protein determination. Protein concentration in synaptic membrane particulates was determined by a modification of the Lowry method [17] of micro-protein analysis.

Electron microscopy. Cell fractions were prepared for electron microscopy by first fixing a synaptic membrane suspension (500 μg) for 5 hr with Ito–Karnovsky's fixative, and then post-fixing with cold 1% OsO_4 in cacodylate buffer for 2 hr. Membranes were sequentially dehydrated with 30 to 100% ethanol, washed with propylene oxide and treated overnight with 100% Epon at room temperature. The pellet was placed in BEEM capsules containing 100% Epon, heated at 60° for 2 hr, and sectioned. Silver to grey sections were mounted on 200-mesh copper grids and stained with ethanolic uranyl acetate for 20 min and lead citrate for 2 min. Sectioning and electron microscopic examinations were performed by Dr. Penelope Fenner-Crisp, Dept. of Anatomy, Georgetown University, and by Drs. Gunther Bahr and R. A. Shoemaker, Armed Forces Institute of Pathology.

RESULTS

Morphology of synaptic membrane preparations. The heterogeneity of both DeRobertis and Whittaker synaptic membrane suspension was clearly visible in electron micrographs (Figs 1A and 1B); however, the greater purity of the Whittaker preparation is evident. Synaptosomes, synaptic vesicles, myelin and axon fragments, mitochondria, and unidentified membrane fragments were all present in the DeRobertis membranes, in addition to the fused and unfused synaptic membranes. The Whittaker preparations appeared to consist almost entirely of sheets of membranes, the ends of which being "fused" to form empty vesicles, along with unfused membranes. Significant numbers of presynaptic storage vesicles were also observed, although far fewer than seen in the DeRobertis preparations.

General properties of dopamine binding. Specific dopamine binding to calf corpus striatal synaptic membranes prepared by the Whittaker procedure was saturable as determined from concentration-dependent competition curves (Fig. 2). Half-maximal levels of dopamine binding were attained at concentrations of about 5.5 μM, as derived from Klotz double reciprocal plots of the competition data (curves not shown). Specific dopamine binding was also linear up to 0.27 mg/ml synaptic membrane protein with Whittaker preparations. Calf striatal synaptic membranes bound [1-^{3}H]dopamine half-maximally within 15 to 30 min of initiation of the binding reaction (Fig. 3). Temperature changes between 0° and 30° did not significantly influence the binding of dopamine to synaptic membranes; however, binding increased sharply at physiological (37°) and higher temperatures. Specific dopamine binding to Whittaker calf striatal synaptic membranes was very low at pH 7.0 but steadily increased to a maximum at pH 7.8, beyond which specific binding rapidly declined. Dithiothreitol (0.1 mM), an antioxidant, reduced specific dopamine binding to synaptic membranes by about 30 per cent, suggesting that air oxidation of dopamine to quinones may produce some artifactual binding if not controlled. Quinone products of catecholamine oxidation have been shown to bind rapidly, covalently, and non-specifically to plasma membrane proteins (Saner

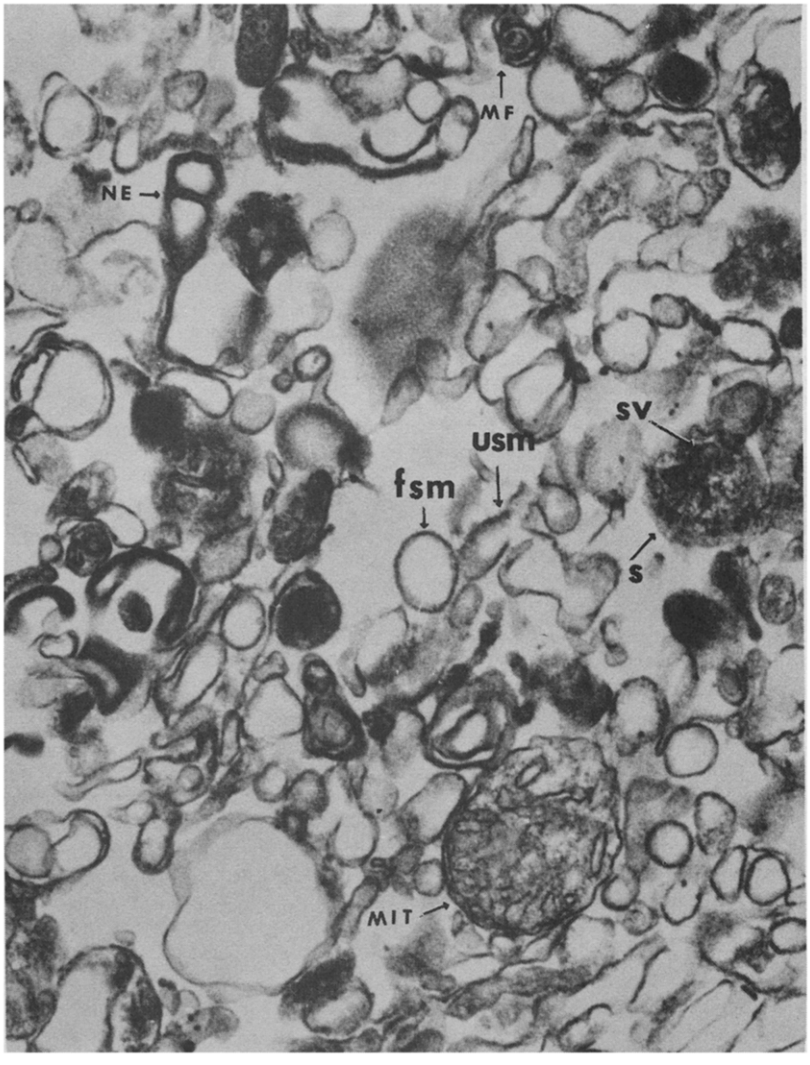

Fig. 1(A).

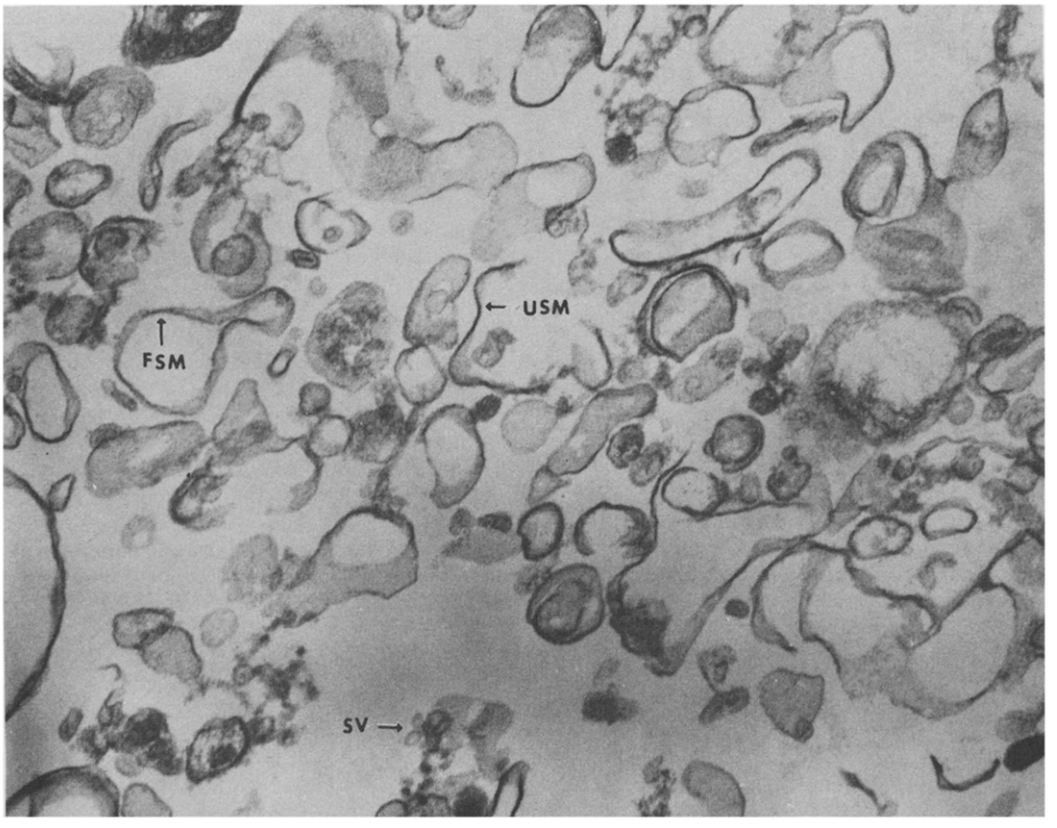

Fig. 1(B).

Fig. 1(A). Electron micrograph of DeRobertis rat brain cortical synaptic membrane fraction. Synaptic membrane fractions were prepared for electron microscopic examination by Dr. Penelope Fenner-Crisp, Georgetown University, as described in Methods. Examination of the DeRobertis rats brain cortical synaptic membrane sections (×62,500) revealed the following: shrunken nerve endings (NE); synaptosomes (S); fused synaptic membranes (FSM); unfused synaptic membranes (USM); mitochondria (MIT); storage vesicles (SV); and myelin figures (MF). (B) Electron micrograph of Whittaker calf brain striatal synaptic membrane fraction. Synaptic membrane fractions were prepared for electron microscopic examination by Dr. Robert E. Shoemaker, Armed Forces Institute of Pathology as described in Methods. Examination of the Whittaker calf brain striatal synaptic membrane sections (×63,260) revealed the following: fused synaptic membranes (FSM); unfused synaptic membranes (USM); and storage vesicles (SV). No mitochondria were apparent.

and Theonen [18]). It should be noted that the reduction of binding due to DTT could also have resulted from the reduction of disulfide bonds by DTT, assuming that such bonds are involved with dopamine receptors.

Metabolism of dopamine in synaptic membrane preparations. Low-to-undetectable activities of dopamine metabolizing enzymes were found in Whittaker synaptic membrane preparations; higher activities were found in DeRobertis preparations (Table 1). Monoamine oxidase, which is localized within outer mitochondrial membranes, could theoretically oxidize as much as 30 per cent of the [1-^{3}H]dopamine present with 50 μg of DeRobertis rat cortical synaptic membrane protein during a 2 hr binding period in the presence of ideal concentrations of cofactors; in contrast, no more than 5 per cent of the [1-^{3}H]-dopamine would be theoretically metabolized by the MAO contained in 25 μg of Whittaker calf striatal synaptic membrane preparations. Dopamine β-hydroxylase, localized in storage vesicles of adrenergic nerve terminals, was not detectable in any Whittaker synaptic membrane preparation; however, up to 15 per cent of the total [1-^{3}H]dopamine added to 50 μg of DeRobertis rat cortical synaptic membrane protein could be theoretically converted to norepinephrine in a 2 hr period in the presence of ideal concentrations of Cu^{2+} and other cofactors. Catechol-*O*-methyl transferase, localized in the cleft of synaptic junctions and in the cytosol of nerve terminals, was virtually undetectable in Whittaker calf preparations, but significant amounts could be detected in DeRobertis synaptic membranes in the presence of excess *S*-adenosyl methionine and other cofactors.

These results suggest that, whereas significant amounts of dopamine could theoretically be metabolized by enzymes contained in DeRobertis preparations, very little, if any, metabolism of [1-^{3}H]dopamine would occur with Whittaker synaptic membranes.

Reversibility and product characterization. Dopamine binding to Whittaker calf striatal synaptic membranes was partially reversible. "Chase" techniques, in which a large excess of unlabeled dopamine was added to membranes prelabeled with [1-^{3}H]dopamine, displaced about 43 per cent of the bound counts

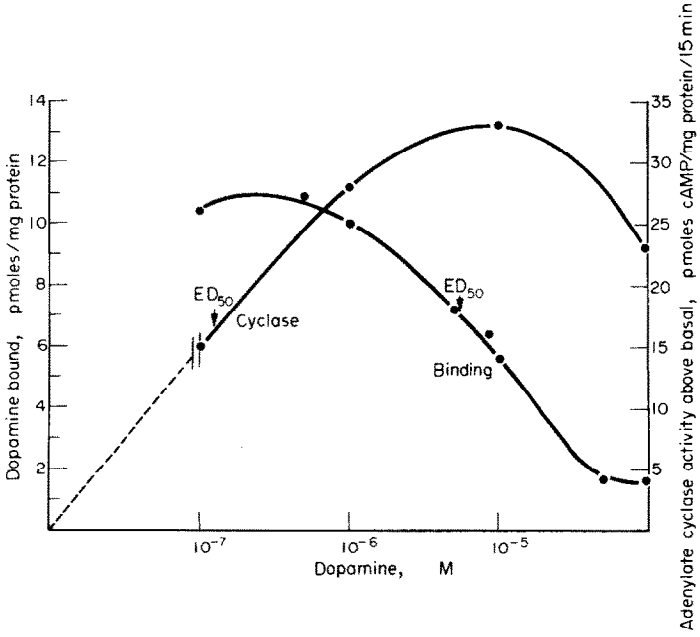

Fig. 2. Dopamine-stimulatable adenylate cyclase activity and concentration-dependent binding of [1-^{3}H]dopamine in Whittaker calf brain striatal synaptic membranes. Adenylate cyclase was assayed in 43 μg of synaptic membrane protein, as described in Methods; basal activity was 83 pmoles cAMP produced per mg protein in 15 min. Dopamine binding was assessed in 29 μg of synaptic membrane protein as described in Methods.

(Fig. 3). In parallel experiments, 93 per cent of bound ^{3}H-ligand was extracted from membranes with HCl and chromatographed as described in Methods. All extracted radioactivity was found in a single spot with an R_f (0.44) identical with that of authentic dopamine; no other radioactive materials were detected (Fig. 4).

Pharmacological. Contamination of synaptic membrane preparations with storage vesicles, as suggested by electron microscopy, was confirmed by both bio-

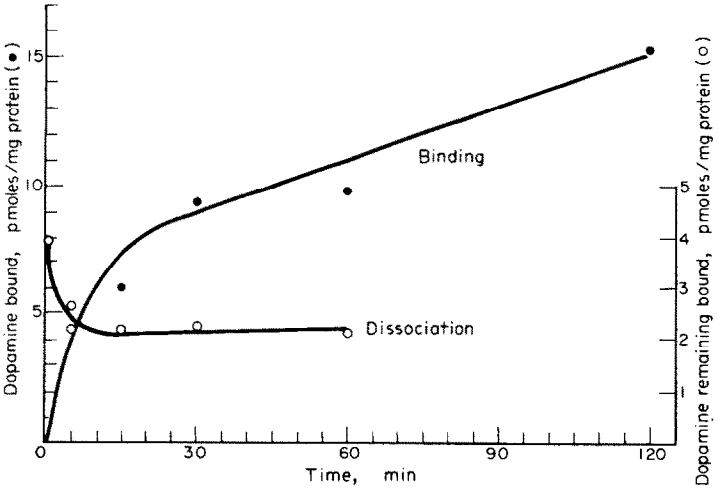

Fig. 3. Time dependence of binding and dissociation of dopamine with Whittaker calf brain striatal synaptic membranes. Incubation mixtures for binding studies contained [1-^{3}H]dopamine (0.1 M) and synaptic membranes (30 μg protein) in 50 mM Tris-HCl buffer (pH 7.8), in a final volume of 150 μl. Incubations, conducted at 37°, were ended at the times shown in the figure. For dissociation experiments synaptic membranes (135 μg protein), 0.1 mM ascorbic acid, 50 mM Tris-HCl buffer (pH 7.8), and [1-^{3}H]dopamine 0.1 μM) were incubated for 2 hr at 37°, after which the membranes were reisolated by centrifugation (39,000 g for 30 min in the Sorval RC-2B). Fresh buffer and ascorbic acid were added to a final volume of 2.85 ml, and the membranes were resuspended. At this time, aliquots (150 μl) were taken to determine initial binding values, after which unlabeled dopamine (0.1 mM) was added to the remaining suspension to begin the chase of ^{3}H-ligand from receptors. These suspensions were further incubated, taking 150 μl aliquots at the times shown in the figure.

Table 1. Assays for dopamine metabolising enzymes*

Enzyme activity (pmoles/min/mg prot)	DeRobertis rat brain cortex	Whittaker rat brain cortex	Whittaker corpus striatum	
			Rat brain	Calf brain
Dopamine β-hydroxylase	0.37	ND	ND	ND
Catechol *O*-methyl transferase	0.13	0.02	0.10	ND
Monoamine oxidase	0.71	0.24	0.18	0.21

* Synaptic membrane preparations were assayed for the activities of the major dopamine-metabolizing enzymes, as described in Methods. ND = not detectable.

chemical and pharmacological criteria. "Chase" experiments, utilizing cations instead of unlabeled dopamine or simple dilution, were used to demonstrate storage vesicle sequestration of [1-^{3}H]dopamine. Calcium ions, which are known to effect the release of catecholamines from storage vesicle sites (Willis and Grossman [19]), stimulated the release of 40 per cent of the pre-bound [1-^{3}H]dopamine from relatively crude DeRobertis rat cortical synaptic membranes over a 90 min chase period; 25 per cent of the pre-bound dopamine was lost from Whittaker calf striatal synaptic membranes in a parallel study. On the other hand, magnesium, which is known to inhibit Ca^{2+}-stimulated catecholamine release from storage sites (Willis and Grossman [19]), slightly *increased* the amount of dopamine apparently bound to both DeRobertis and Whittaker synaptic membranes in chase experiments performed as above.

The effects of reserpine on dopamine binding reactions using Whittaker calf striatal synaptic membranes also suggested contamination with storage vesicles. Reserpine is a known inhibitor of synaptic vesicle reuptake processes (Koelle [20]), and is suspected of interfering with receptor sites linked to adenylate cyclase (Zivkovic [21]). High concentrations of this drug (0.1 mM) inhibited the binding of [1-^{3}H]dopamine by 77 per cent, whereas low concentrations (0.1 μM) decreased binding by only 22 per cent (Fig. 5).

Pargyline, an MAO inhibitor (Koelle [20]), when present in dopamine binding reactions using Whittaker calf striatal synaptic membranes, was without significant effect (Fig. 5), supporting earlier findings (Table 1) of negligible contamination with this enzyme.

The effects of cocaine on dopamine binding to Whittaker calf striatal synaptic membranes were assessed in order to quantify binding to presynaptic membrane fragments or synaptosomal membranes as part of an active reuptake system (Koelle [20]). A maximum inhibition of about 34 per cent of total dopamine binding was observed, suggesting that about one-third of all specific dopamine binding occurs in association with some presynaptic binding systems (Fig. 5).

Finally, the degree of dopamine binding to specific post-synaptic adenylate cyclase-linked receptors was determined utilizing the potent dopamine antagonist, fluphenazine. A maximum inhibition of 44 per cent of dopamine binding to Whittaker calf striatal synaptic membranes occurred (Fig. 5), thus confirming the presence in these synaptic membrane preparations of substantial concentrations of postsynaptic adenylate cyclase-linked dopamine receptors.

Relationship of dopamine binding to activation of adenylate cyclase. Very little dopamine-responsive adenylate cyclase activity was found in synaptic membranes prepared from rat brain cortex. When crude

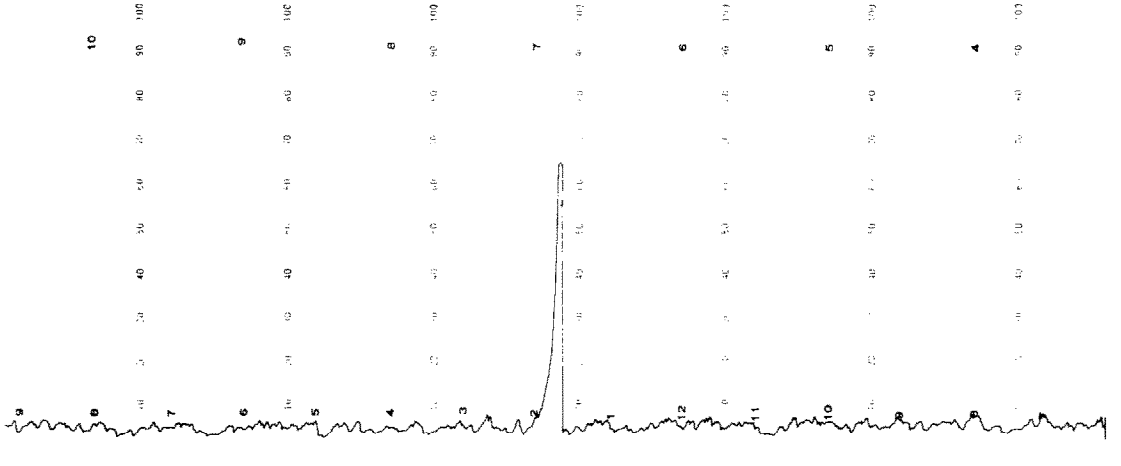

Fig. 4. Chromatographic characterization of labeled material bound to Whittaker calf brain striatal synaptic membranes. [1-^{3}H]Dopamine (0.1 μM) was incubated with 0.1 mM ascorbic acid and synaptic membranes (148 μg protein) in 50 mM Tris–HCl buffer (pH 7.8) in a final volume of 1.0 ml. Incubations were carried out for 2 hr at 37°, and membrane-bound labeled material was extracted with HCl as described in Methods. This material was analyzed by paper chromatography as described in Methods. The single peak appearing (R_f = 0.44) was identified as dopamine by reference to a standard.

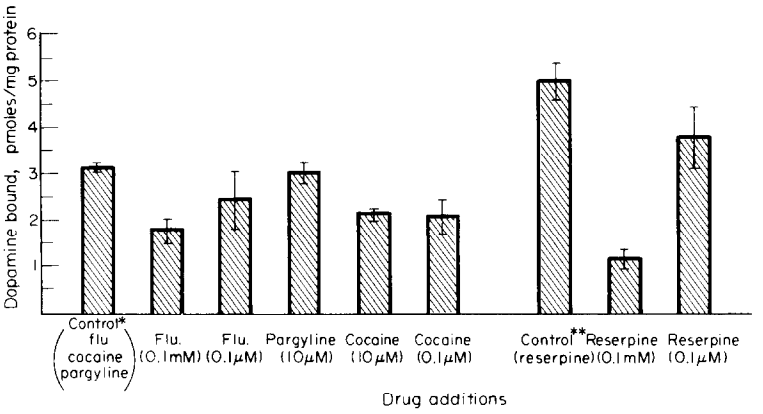

Fig. 5. The effect of drugs which interfere with dopamine metabolism and binding on the specific binding of dopamine to Whittaker calf brain striatal synaptic membranes. Binding of [1-^{3}H]dopamine (0.1 μM) to synaptic membranes (56 μg protein) was carried out for 1 hr in the presence of fluphenazine, pargyline, cocaine, or reserpine, at the concentrations shown in the figure, in glycylglycine buffer (50 mM, pH 8.0), final volume of 150 μl. Control tubes for fluphenazine, cocaine, and pargyline contained buffer in place of drugs, while the control tubes for reserpine contained its solvent, 1 mM sodium acetate, pH 7, in place of drug. Mixtures were preincubated for 1 hr prior to the addition of [^{3}H]dopamine.

homogenates of cortex were converted to synaptic membranes by either the DeRobertis or Whittaker methods, the slight sensitivity of adenylate cyclase activity to dopamine essentially disappeared; however, some cyclase response to dopamine could be elicited in such synaptic membranes by the addition of the nucleotides GTP or 5′-guanylyl imidodiphosphate, which have been reported (Cuatrecasas *et al.* [22]) to facilitate the activation of adenylate cyclases by catecholamines. In contrast, rat and calf striatal synaptic membranes prepared by the Whittaker procedure contained an adenylate cyclase which was stimulatable by dopamine; maximum stimulatability of the rat enzyme was about 140 per cent, but only about 40 per cent in the case of the calf preparation.

The ED_{50} values for dopamine binding to Whittaker rat and calf striatal synaptic membranes were 4.4 μM and 5.4 μM, respectively. These concentrations are over an order of magnitude greater than for dopamine activation of adenylate cyclase in these preparations (ED_{50}, 0.12, Table 2, Fig. 4), which suggests that extraneous acceptors for dopamine may be present in these membrane preparations. Scatchard plots from competition binding experiments suggested the existence of two classes of binding sites, with the affinity of dopamine for the second class of sites being very low (Table 3).

The concentration of dopamine which bound half-maximally in the presence of 10 μM fluphenazine (ED_{50}, 3.7 μM, Table 2) was essentially the same as in controls; however, this drug completely abolished dopamine activation of adenylate cyclase. Although fluphenazine did not change significantly the association constant of dopamine for the higher affinity sites (Table 3), it reduced the affinity of dopamine for the second class of sites to an unmeasurable value.

Reserpine (100 μM) also abolished dopamine activation of adenylate cyclase (Table 2), and it inhibited specific binding of dopamine to Whittaker calf striatal synaptic membranes to such a large extent that

Table 2. Effects of psychoactive drugs on the ED_{50} for dopamine binding and activation of adenylate cyclase in synaptic membrane preparations isolated from calf brain corpus striatum according to Whittaker*

Additions	ED_{50}	
	Dopamine binding	Activation of adenylate cyclase
	μM	
Control	5.4	0.12
Fluphenazine, 10 μM	3.7	no activation
Pargyline, 10 μM	2.9	0.42
Reserpine, 100 μM	not measurable	no activation
Cocaine, 100 μM	4.2	0.66

* Assays for dopamine-stimulated adenylate cyclase were performed as described in Fig. 2, in the absence and presence of the drugs shown above; ED_{50} values are defined as the concentration of dopamine required to stimulate adenylate cyclase half-maximally. Binding curves were developed from competition experiments carried out as described in Fig. 2 in the absence and presence of the drugs listed above; ED_{50} values are defined as the concentration of dopamine required for half-maximal binding.

Table 3. Effects of psychoactive drugs on the apparent affinity constants for dopamine binding to Whittaker calf brain corpus striatum synaptic membranes*

Additions	Ka_1	Ka_2
	μM^{-1}	mM^{-1}
Control	0.10	0.10
Fluphenazine (10 μM)	0.15	†
Pargyline (10 μM)	0.21	†
Reserpine (10 μM)	–	–
Cocaine (100 μM)	0.29	–

* Scatchard plots were made from data provided by competition experiments performed as described in Fig. 2. Apparent affinity constants (Ka_1 and Ka_2) were calculated from the slopes of the curves obtained by plotting B/F ratios against B, for the binding of [1-^{3}H]dopamine. B, pmoles dopamine bound, is the product of B/F and the total pmoles of dopamine added to the incubation mixture.

† Affinity too low to calculate association constant.

neither an ED_{50} (Table 2) nor an association constant for either class of binding sites (Table 3) could be determined.

The ED_{50} value of 4.2 μM for dopamine binding in the presence of cocaine was much the same as in controls (Table 2); however, the ED_{50} for the activation of adenylate cyclase in the presence of cocaine (0.66 μM) was about five times that of controls. This may reflect a direct interference of cocaine with dopamine activation of the cyclase. Scatchard analysis of binding data obtained in the presence of cocaine revealed only a single class of binding sites with an association constant of 0.29 μM^{-1} (Table 3).

Pargyline was without significant influence on the ED_{50} and K_{a1} values for dopamine binding (Tables 2 and 3) or on the activation constant for adenylate cyclase (Table 2), but it reduced the affinity of dopamine to the second class of binding sites to unmeasurable values (Table 3).

Maximal non-specific binding of dopamine to Whittaker calf striatal synaptic membranes as determined from competition studies was 25 per cent in control studies (Table 4). Cocaine and reserpine essentially eliminated non-specific binding of dopamine, suggesting that they not only block active reuptake systems when utilized at high concentrations, but non-specifically block postsynaptic adenylate cyclase-linked receptors as well. On the other hand, fluphenazine *increased* non-specific binding, supporting the existence of specific postsynaptic adenylate cyclase-linked dopamine receptors (Table 4). Pargyline apparently caused no significant change in non-specific binding (Table 4).

Table 4. Effects of psychoactive drugs on the nonspecific binding of dopamine by Whittaker preparations of calf brain striatal synaptic membranes*

Additions	Non-specific binding
	%
Control	25
Fluphenazine (10 μM)	42
Pargyline (100 μM)	16
Reserpine (100 μM)	0
Cocaine (100 μM)	0

* Competition binding experiments between fixed concentration of [1-^{3}H]dopamine (0.1 μM) and varied concentrations of unlabeled dopamine (0.1 μM–0.1 mM) were carried out as described in Fig. 2 in the absence and presence of the drugs shown below. Nonspecific binding of dopamine is defined as the percent of [1-^{3}H]dopamine which remained bound in the presence of 0.1 mM of unlabeled dopamine.

DISCUSSION

Morphological and biochemical assessments of two widely-used preparations of mammalian brain synaptic membranes have clearly established the greater purity of the Whittaker preparation, compared to that of DeRobertis. Enzyme assays and electron microscopy revealed that the latter, but not the former, was heavily contaminated with dopamine metabolizing enzymes, with synaptic storage vesicles, and with extraneous neuronal membranes.

Rat cortical synaptic membranes prepared by either method contained dopamine binding sites and adenylate cyclase; however, the cyclase of neither cortical preparation was stimulated by dopamine. Rat and calf striatal membranes also contained dopamine acceptors and adenylate cyclase activity; in this case, however, the enzyme *was* stimulatable by dopamine.

The binding of dopamine to membrane sites in Whittaker calf striatal preparations exhibited many of the characteristics of specific receptors; that is, it was relatively rapid, saturable, antagonized by compounds known to act similarly *in vivo*, unaffected by dopamine-metabolizing enzymes, and was, apparently, noncovalent under our incubation conditions. Binding of dopamine was, however, only partially reversible in chase experiments with high concentrations of unlabeled ligand; this behavior, as well as the release of dopamine in the presence of Ca^{2+}, suggested that a substantial portion of the membrane-associated [1-^{3}H]dopamine may have been the result of unidirectional sequestration, perhaps involving storage granules.

In our hands, concentrations of dopamine required for half-maximal binding to Whittaker striatal membrane preparations (ED_{50}, 5.4 μM) were over an order of magnitude greater than those necessary for half-maximal activation of adenylate cyclase (ED_{50}, 0.2 μM). This is not surprising in view of our findings that about 55–60 per cent of total dopamine binding was to non-postsynaptic membrane sites. Our pharmacological and kinetic evidence suggests that there are at least two types of such additional sites in Whittaker striatal membrane preparations: about one-third of total dopamine binding may be due to interaction of dopamine with a presynaptic reuptake system, and about one-fourth of total dopamine binding may occur as a result of sequestration by presynaptic storage vesicles.

Direct assessment of the activities of dopamine-metabolizing enzymes in Whittaker calf striatal synaptic membrane preparations suggests that these enzymes are not factors contributing to total dopamine binding. These measurements, coupled with the chromatographic evidence that [1-^{3}H]dopamine is not chemically altered by contact with the binding

system and with our evidence for specific inhibition by fluphenazine of dopamine binding to postsynaptic anedylate-cyclase-linked receptors, refute the suggestions of Cuatrecases *et al.* [23] that catecholamine binding to various membrane preparations represents simple substrate interaction with catechol-*O*-methyl transferase or non-specific binding of quinones to membrane proteins, and the suggestion of Lindl and Cramer [24] that dopamine is converted to norepinephrine which is the binding species.

Burt *et al.* [25] and Seeman *et al.* [26], in reports which appeared during the present studies, described the binding of dopamine and psychoactive drugs to preparations derived from rat and calf corpus striatum. The former group used a 50,000 *g* particulate fraction derived from a simple homogenate of stored (at −20°) tissue, in which specific binding of [^{3}H]-dopamine to this preparation was only about 20 per cent of total binding, compared to 75 per cent in the present studies. Seeman *et al.* [26] used a preparation of synaptosomes. On the basis of a good correlation between the pharmacological and binding potencies of catecholamines and neuroleptic drugs and of an unchanged binding of dopamine between striatal preparations with and without lesions of the nigro-striatal dopamine pathways with 6-hydroxydopamine, Burt *et al.* [25] concluded that the binding of dopamine and psychoactive drugs was exclusively to the postsynaptic receptors in their particulate preparations. In addition, Burt *et al.* [25], reporting an IC_{50} value of 0.38 μM for dopamine binding to rat brain particulates, used literature values to calculate IC_{50} values of 2-to-5 μM for dopamine activation of adenylate cyclase in their preparation; on this basis, the authors inferred that the affinity of dopamine for its binding site is greater than its potency in activating adenylate cyclase. We question these conclusions for several reasons, chief among which are: (i) they are based upon binding constants determined with a very crude brain preparation in which we find that dopamine can bind to at least three different neuronal membrane components, as well as to three dopamine-metabolizing enzymes; (ii) presynaptic dopamine receptors in the brain have been previously described (Roth *et al.* [27]; Iverson *et al.* [28]); and (iii) our reserpine/cocaine experiments demonstrate that even in the more-pure Whittaker striatal preparations substantial contributions to total dopamine binding are made by presynaptic elements. Similarly, Seeman *et al.* [26], in studying the stereospecific binding of [^{3}H]dopamine and [^{3}H]haloperidol to rat brain striata, used the relatively crude synaptosomal preparation in attempts to establish that antipsycholtic drugs inhibit the specific binding of dopamine to extents which are correlatable with their clinical potency. In view of the structural heterogeneity of the tissue preparation employed and in the face of the very low extents of dopamine binding encountered by these workers, we feel that no conclusions can be drawn from these studies as to the site of antipsychotic action of the drugs studied.

We suggest that the satisfactory characterization of the binding of dopamine and psychoactive drugs to striatal sites must await the development of better isolation techniques for pre- and post-synaptic membranes and synaptic junctional complexes.

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