

Dihydropyridine, a Novel Full Efficacy D₁ Dopamine Receptor Agonist¹

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ABSTRACT

The present work provides a detailed pharmacological characterization of dihydropyridine (DHP) (*trans*-10,11-dihydroxy-5,6,6a,7,8,12b-hexahydrobenzo[*a*]phenanthridine), the first high-potency, full efficacy, bioavailable D₁ dopamine receptor agonist. DHP represents a new conformationally rigid structural class of dopamine receptor ligands. It competes stereoselectively and potently for D₁ binding sites in rat striatal membranes labeled with [³H]SCH23390 with an IC₅₀ of about 10 nM compared to about 30 nM for the prototypical D₁ agonist SKF38393. Like other dopamine agonists, DHP has a shallow competition curve (*n*_H = *ca.* 0.7) that can be fitted by a two-site model consisting of high-affinity (63%; *K*_D = 3 nM) and low-affinity (37%; *K*_D = 75 nM) sites. DHP was screened for activity against 40 other binding sites, and was inactive (IC₅₀ >10 μM) against all except D₂ dopamine receptors (IC₅₀ = 130 nM) and α₁ adrenoreceptors (IC₅₀ = *ca.* 230 nM). Functionally, DHP is a full efficacy dopamine

D₁ agonist. In homogenates of rat striatum, DHP or dopamine doubles the rate of cyclic AMP synthesis, whereas SKF38393 only causes a maximal increase of about 50%. These effects of DHP are blocked by the selective D₁ antagonist SCH23390, but are not affected by D₂, 5-hydroxytryptamine₂, muscarinic, or α or β adrenergic antagonists. Because DHP is known to cause D₂-like behavioral effects at high doses, the nature of its D₂ activity was characterized using prolactin release as an endpoint. DHP and the prototypical D₂ agonist quinpirole both caused a significant inhibition of the prolactin release induced by 5-hydroxytryptophan. These effects of DHP are not due to "indirect" alterations at the presynaptic terminal, because DHP is essentially inactive at inhibiting the dopamine uptake system, and does not cause the release of dopamine. These data demonstrate the utility of DHP for probing the biochemistry and function of D₁ dopamine receptors.

Traditionally, dopamine receptors have been divided into two major subclasses (D₁ and D₂) based on pharmacological and functional data (Kebabian and Calne, 1979). Molecular cloning studies have now shown that the D₁ subclass (which we term "D₁-like") consists of several distinct molecular forms including D_{1a} (Dearry *et al.*, 1990; Zhou *et al.*, 1990; Sunahara *et al.*, 1990; Monsma *et al.*, 1990; Gingrich *et al.*, 1991), D_{1b} (Tiberi *et al.*, 1991) and D₈ (Sunahara *et al.*, 1991), although this area is still controversial. The D₂ subclass (which we term "D₂-like") consists of at least four forms: D_{2long} and D_{2short} (Bunzow *et al.*, 1988; Eidne *et al.*, 1989; Giros *et al.*, 1989; Monsma *et al.*, 1989); D₃ (Sokoloff *et al.*, 1990); and D₄ (Van

Tol *et al.*, 1991). In general, D₁-like receptors are often linked functionally to stimulation of cAMP synthesis, and preferentially recognize 1-phenyl-tetrahydrobenzazepines (*e.g.*, SCH23390) over benzamides (*e.g.*, sulpiride). There may be other members of the D₁-like class with similar pharmacology that are not coupled to adenylate cyclase (Mailman *et al.*, 1986). The D₂-like receptors (D_{2long}, D_{2short}, D₃ and D₄) often are linked to inhibition of cAMP synthesis, and have the opposite pharmacological specificity (*i.e.*, preferring sulpiride over SCH23390). Within each major group, however, there are important differences in drug recognition (*e.g.*, higher affinity of clozapine for the D₄ vs. D₂ or D₃ forms).

Although these molecular studies have provided important information about dopamine receptor subtypes, their functional role in the nervous system is still not well understood, in part due to the lack of subtype-selective ligands. For example, the

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ABBREVIATIONS: DHP, dihydropyridine (*trans*-10,11-dihydroxy-5,6,6a,7,8,12b-hexahydrobenzo[*a*]phenanthridine); HTP, 4-(3,4-dihydroxyphenyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine; BrMe-DHP, *trans*-10-bromo-11-hydroxy-6-methyl-5,6,6a,7,8,12b-hexahydrobenzo[*a*]phenanthridine; Ro 21-7767/001, (*trans*-6-methyl-9,10-dihydroxy-5,6,6a,7,8,12b-hexahydrobenzo[*a*]phenanthridine); SKF82958, 6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; 5-HTP, 5-hydroxytryptophan; cAMP, cyclic AMP; HEPES, N-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid; *n*_H, Hill coefficient; UV, ultraviolet; KB, Krebs' buffer; RIA, radioimmunoassay; NMDA, N-methyl-D-aspartate; PCP, phencyclidine; GABA, gamma-amino-butyric acid; ANF, atrial natriuretic factor; EGF, epidermal growth factor; NGF, nerve growth factor; NPY, neuropeptide Y; VIP, vasoactive intestinal polypeptide.

functional importance of D₁-like receptors in the central nervous system was largely misunderstood until the selective D₁ antagonist SCH23390 (Iorio *et al.*, 1983) became available. It rapidly became clear that D₁ receptor antagonists could affect D₂-mediated behaviors (Mailman *et al.*, 1984; Christensen *et al.*, 1984), leading to a focus on D₁/D₂ receptor interactions (*e.g.*, see Clark and White, 1987).

Unfortunately, functional studies of D₁ receptors have been impeded by the characteristics of the available agonists. Although several high-affinity D₁ antagonists of the phenyltetrahydrobenzazepine class are known (*e.g.*, SCH23390 or SCH23982), the prototypical agonists (*e.g.*, SKF38393, fenoldopam) also are based on this same chemical backbone. SKF38393 and its analogs are relatively potent agonists, but are only partially efficacious (relative to dopamine) in stimulating adenylate cyclase. For example, SKF38393 causes only 30 to 50% as much increase in cAMP synthesis in rat striatum or cultured cell lines as does dopamine (*e.g.*, Brewster *et al.*, 1990; Lovenberg *et al.*, 1991). Moreover, it is unclear whether the modest psychopharmacological effects produced by SKF38393 are a result of the partial efficacy of this drug, or of the intrinsic limits of activation of D₁ receptors. Thus, results of functional studies using SKF38393 may not reflect actual consequences of D₁ receptor occupation and activation. Such factors have led to our focus on the rational development of new D₁ receptor ligands, especially those that would be full efficacy agonists.

We have provided preliminary reports about the synthesis of DHX (see fig. 1) and its potential as the first high-potency, full efficacy, bioavailable D₁ agonist (Lovenberg *et al.*, 1989; Brewster *et al.*, 1990). In the present work, we provide detailed pharmacological characterization of DHX, a member of a new class of rigid dopaminergic ligands, the benzo[a]phenanthridines. The design of these drugs was based on a model (Nichols, 1983) that predicted that these conformationally rigid compounds would be effective D₁ agonists due to their "β-phenyl-

dopamine pharmacophore" and the elimination of the free rotation of the accessory phenyl ring (*e.g.*, see Charifson *et al.*, 1989; Brewster *et al.*, 1990). Recent data have shown that DHX has unique psychopharmacological effects in both rats (Darney *et al.*, 1991) and monkeys (Elsworth *et al.*, 1991; Taylor *et al.*, 1991). The present article characterizes the pharmacological activity of DHX and provides data showing its potent functional activity at D₁ receptors, its modest activity at D₂ receptors and its overall selectivity for dopamine receptors. Moreover, the data also demonstrate that these actions are mediated directly at dopamine receptors, and not *via* indirect actions caused either by inhibition of uptake or increased release of dopamine. These pharmacological characteristics demonstrate that DHX should be a valuable tool for the study of multiple aspects of D₁ receptor function and the interaction of D₁ and D₂ receptors.

Materials and Methods

Materials

[³H]SCH23390 (ca. 70 Ci/mmol) was synthesized as described by Wyrick and Mailman (1985). [³H]Spiperone was purchased from Amersham Corp. (Arlington Heights, IL). [α-³²P]ATP, [³H]dopamine, [³H]serotonin and Na ¹²⁵Iodine were supplied by New England Nuclear (Boston, MA) and HEPES buffer was purchased from Research Organics, Inc. (Cleveland, OH).

SCH23390 was a gift from Schering Corp. (Bloomfield, NJ) or was purchased from Research Biochemicals Inc. (Natick, MA). SKF82958, SKF38393 and phenoxybenzamine were gifts from Smith Kline and French (Philadelphia, PA); some SKF38393 was purchased from Research Biochemicals Inc. (Natick, MA). Quinpirole (LY171555) and fluoxetine were gifts from Lilly Research Laboratories (Indianapolis, IN). The compound Ro 21-7767/001 was a gift from Hoffmann La-Roche Inc. (Nutley, NJ). Isobutyl methylxanthine, cAMP, dopamine, serotonin, DL-propranolol, (-)-sulpiride, chlorpromazine, diazepam, atropine and 5-HTP were purchased from Sigma Chemical Co. (St. Louis, MO). Spiperone, ketanserin and domperidone were gifts from Janssen Pharmaceutica (Beerse, Belgium and New Brunswick, NJ). GBR-12909 was a generous gift from Novo Industries (Bagsvaerd, Denmark). cAMP primary antibody was obtained from Dr. Gary Brooker, and secondary antibody was a rabbit anti-goat immunoglobulin G (BioRad, Richmond, CA). DHX, its analogs and THP were synthesized using methods that have been published previously (Truex *et al.*, 1985; Brewster *et al.*, 1990; Brewster, 1990).

Tissue Preparation

Male Sprague-Dawley rats weighing 200 to 400 g were decapitated, and the brains quickly removed and placed into ice-cold saline. After a brief chilling period, brains were sliced into 1.2-mm coronal slices with the aid of a dissecting block similar to that described by Heffner *et al.* (1980). The striatum was dissected from two slices containing the majority of this region, and the tissue was either used immediately or stored at -70°C until the day of the assay.

Radioligand Receptor Binding

[³H]SCH23390-labeled D₁ receptors. Radioligand binding followed the method of Schulz *et al.* (1985) with minor modifications. After dissection, rat striata were homogenized by seven manual strokes in a Wheaton Teflon-glass homogenizer with ice-cold 50 mM HEPES buffer with 4.0 mM MgCl₂, pH 7.4 (25°C). Tissue was centrifuged at 27,000 × g (Sorvall RC-5B/SS-34 rotor, DuPont, Wilmington, DE) for 10 min, and the supernatant was discarded. The pellet was homogenized (five strokes) and resuspended in ice-cold buffer and centrifuged again. The final pellet was suspended at a concentration of approximately 2.0 mg wet wt./ml.

Assay tubes (1 ml final volume) were incubated at 37°C for 15 min.

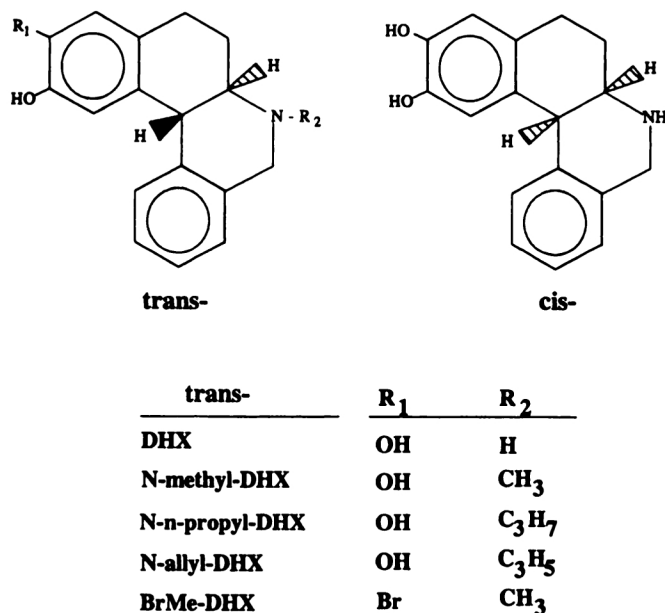


Fig. 1. Structure of DHX and several analogs. This figure shows the structure of DHX, several DHX analogs, as well as the inactive diastereomer *cis*-10,11-dihydroxy-5,6,6a,7,8,12b-hexahydrobenzo[a]phenanthridine.

Nonspecific binding of [³H]SCH23390 (ca. 0.25 nM) was defined by adding unlabeled SCH23390 (1 μ M). Binding was terminated by filtering with 15 ml of ice-cold buffer on a Skatron or Brandel cell harvester (Skatron Inc., Sterling, VA; Brandel Inc., Gaithersburg, MD) using glass fiber filter mats (Skatron no. 7034; Brandel GF/B). Filters were allowed to dry, and 2 to 4 ml of Scintiverse E (Fischer Scientific Co., Fair Lawn, NJ) were added. After shaking for 30 min, radioactivity was determined on an LKB-1219 RackBeta liquid scintillation counter. Tissue protein levels were estimated using the Folin reagent method of Lowry *et al.* (1951) adapted to a Technicon Autoanalyzer I (Tarrytown, NY).

[³H]Spiperone-labeled D₂ receptors. The binding procedure and protein analysis were identical to that described for [³H]SCH23390 binding. Nonspecific binding of [³H]spiperone (ca. 0.07 nM) was defined by adding unlabeled chlorpromazine (1 μ M). Ketanserin tartrate (50 nM) was used to mask binding of [³H]spiperone to serotonin receptors.

Nondopamine receptors. Competition curves were run for several additional receptor types using methods previously described. These assays utilized [³H]prazosin (α_1 adrenergic; Herr and Mailman, 1989), [³H]atipamizole (α_2 adrenergic; Bishop *et al.*, 1991) and [³H]ketanserin (5-hydroxytryptamine; Bishop *et al.*, 1991). The remaining receptor assays were performed by the Nova Pharmaceutical Corporation (Baltimore, MD) using their PROFILE Novascreen program, as generously provided via a contract with the National Institute of Mental Health. This consisted of testing DHX at 0.1 and 10 μ M concentrations against the ligands listed in table 3. In a few of these assays, some competition (<50%) was seen at 10 μ M, but none at 0.1 μ M. Assuming competitive kinetics, it was estimated that the IC₅₀ of DHX in these situations was greater than 10 μ M.

The initial data analysis for the D₁ and D₂ receptor binding competition experiments used customized Lotus spreadsheets to calculate specific binding and provide initial graphical presentation. The data were also analyzed using Hill plots to determine IC₅₀ values and n_H. To analyze the complex binding activity of DHX, nonlinear regression of the binding data was performed using the algorithm in the curve-fitting program InPlot (Graphpad Inc., San Diego, CA). Although this program was adequate in the present case (see under "Results"), the output from InPlot also was used as initial estimates for LIGAND (McPherson, 1983). The resulting output from LIGAND was essentially identical to the input, indicating that InPlot provided a good fit to these data for the two-site model selected.

Dopamine-Sensitive Adenylate Cyclase Assay

The automated high-performance liquid chromatography method of Schulz and Mailman (1984) was used to measure adenylate cyclase activity by separating cAMP from other labeled nucleotides. Briefly, striatal tissue was removed and homogenized at 50 ml/g tissue in 5 mM HEPES buffer (pH 7.5) containing 2 mM ethylene glycol bis(β -aminoethyl ether)N,N'-tetraacetic acid. After eight manual strokes with a Wheaton Teflon-glass homogenizer, an additional 50 ml/g of 100 mM HEPES/2 mM ethylene glycol bis(β -aminoethyl ether)N,N'-tetraacetic acid were added and mixed with one additional stroke. A 20- μ l aliquot of this tissue homogenate was added to a prepared reaction mixture, yielding a final volume of 100 μ l containing 0.5 mM ATP, 0.5 mM isobutyl methylxanthine, [α -³²P]ATP (0.5 μ Ci), 1 mM cAMP, 2 mM MgCl₂, 0.7 mM HEPES buffer, 2 μ M GTP, 0 to 100 μ M dopamine and/or drug, 10 mM phosphocreatinine and 5 U of creatinine phosphokinase. The reaction was initiated by transferring the samples from an ice bath to a water bath at 30°C and terminated 15 min later by the addition of 100 μ l of 3% sodium dodecyl sulfate. Proteins and much of the noncyclic nucleotides were precipitated by the addition of 300 μ l each of 4.5% ZnSO₄ and 10% Ba(OH)₂ to each incubation tube. The samples were centrifuged at 10,000 $\times$ g for 6 min, and the supernatants immediately removed and loaded in an ISIS (Isco Inc., Lincoln, NB) Autoinjector. The high-performance liquid chromatography separations were carried out using a Waters RCM or Z-module equipped with a C18, 10-micron cartridge, using a mobile phase of 150 mM sodium

acetate/20% methanol, adjusted to pH 5.0 with concentrated HCl before filtration (0.2 μ m) and degassing under vacuum. A flow rate of about 4 ml/min was used for separation. The autoinjector was programmed for a 2-min injection interval, with a rinse between samples. A UV detector equipped for 254-nm detection triggered collection of the cAMP fractions via a FOXY fraction collector (ISCO, Inc., Omaha, NE) with a three-way diversion valve. Unlabeled cAMP added to the samples provided the source of UV absorbance and served as an internal standard. Sample recovery was based on UV measurement of total cAMP peak areas quantified using a Nelson 2600 Analytical Chromatography data system (Perkin Elmer Nelson Systems Inc., Cupertino, CA). The radioactivity in each fraction was determined by liquid scintillation spectroscopy.

Prolactin Assay

The inhibition of prolactin release after activation of D₂ dopamine receptors was assayed following the method of Kuhn *et al.* (1981). Briefly, male rats (350–400 g) were injected i.p. with either the serotonin precursor 5-HTP (30 mg/kg) or saline to stimulate an increase in serum prolactin concentrations. Five min later, the rats were injected (i.p.) with DHX (10 mg/kg), quinpirole (10 mg/kg) or 0.1% ascorbic acid vehicle. Rats were placed singly in plastic cages containing wood chips, and sacrificed by decapitation after 25 min. Trunk blood was collected in chilled plastic centrifuge tubes, allowed to clot at 4°C and centrifuged at 1,000 $\times$ g for 15 min at 4°C. Serum was stored at -20°C until the prolactin assay. Prolactin was assayed with reagents and procedures supplied by the NIAMDD Rat Pituitary Hormone Program. The 5-HTP pretreatment group consisted of four rats for each drug condition, whereas the two conditions not receiving 5-HTP (control, DHX 10 mg/kg) represent two rats each. The results from the 5-HTP pretreatment group were analyzed by the InStat statistical package (Graphpad Inc., San Diego CA) using a one-way analysis of variance with *post hoc* pairwise comparisons performed using a *t* test with Bonferroni correction.

cAMP Efflux from Striatal Slices

Superfusion of striatal slices. cAMP efflux from striatal slices followed the method of Stoof and Kebabian (1981) with minor modifications. Briefly, striatal tissue was obtained as described above and sliced further in two directions (90° apart) using a McIlwain tissue chopper (400 μ m setting). The resulting tissue "slices" (i.e., parallel-pipeds) were transferred to a test tube containing chilled, oxygenated KB (121 mM NaCl; 4.84 mM KCl; 1.89 mM CaCl₂; 1.16 mM MgSO₄; 1.17 mM KH₂PO₄; 25 mM NaHCO₃; 0.06 mM ascorbic acid; and 10 mM D-glucose, pH 7.35). Slices subsequently were washed with three 5-ml aliquots of KB and allowed to settle; 35 μ l slices were transferred to each well of the superfusion apparatus described below and washed at 0.1 ml/min for 90 min. The perfusing KB, but not agonist and drug solutions, was oxygenated with 95% O₂/5% CO₂. Base-line aliquots were collected for three 15-min intervals, after which perfusion aliquots were collected in four 10-min intervals. The amount of drug-induced cAMP efflux was expressed as percent of the basal efflux, thereby permitting each chamber to serve as its own control to account for differences in the amounts of tissue present in each of the wells. Three hundred μ l of all samples were assayed in duplicate by RIA.

The superfusion chamber was constructed of Plexiglas (0.953 cm thickness); it consisted of a 15.2 $\times$ 5.1 cm section containing 12 wells (drilled 0.953 cm in diameter and 0.635 cm deep) with a matching lid. These were held tightly together by screws during experiments. Sections of 20-gauge stainless steel tubing served as inlets and outlets in the two well sections. Each well contained a 0.79 cm long section of Tygon tubing into which fritted disks (Kontes, Vineland, NJ; 0.635 cm diameter) were positioned approximately midway to produce an internal volume of approximately 100 μ l. Buffer was pulled through the wells by a peristaltic pump (Ismatec, Cole Palmer, Chicago, IL) via tubing connected to the bottom of each well; incoming buffer entered the wells via polyethylene tubing connected to the lid from six vials.

Both the chamber and supply buffer, or drugs, were maintained at 37°C in a water bath. Samples are collected in 1-ml Technicon sample vials.

cAMP RIA. The concentration of cAMP in superfusion samples was assayed using an RIA of acetylated cAMP as modified from Harper and Brooker (1975). Iodination of cAMP was performed using the method adapted from Patel and Linden (1988). Standard curves were prepared using concentrations of cAMP ranging from 0.625 to 20 pmol/ml, and sample volumes were 300 μ l from superfusion experiments. All samples and standards were acetylated with 10 μ l of a 2:1 solution of triethylamine/acetic anhydride. To these samples, 100 μ l of primary antibody (sheep anti-cAMP, 1:100,000 dilution in 1% bovine serum albumin/50 mM sodium acetate buffer, pH 4.75) and 100 μ l of [125 I] cAMP (40,000 dpm/100 μ l of sodium acetate buffer) were added. Tubes were vortexed and stored at 4°C overnight (ca. 18 hr). Antibody-bound radioactivity was then separated by the addition of 40 μ l of a 1% (w/v, in 1% bovine serum albumin/50 mM sodium acetate buffer) suspension of microspheres coupled to purified rabbit, anti-goat immunoglobulin G, followed by vortexing and further incubation at 4°C for approximately 4 hr. All tubes were centrifuged at 4000 $\times$ g for 20 min. Supernatants were aspirated, and radioactivity in the resulting pellet was determined using an LKB gamma counter coupled to a computer for data analysis. Data were calculated as picomoles of cAMP per milliliter and expressed as percent stimulation above base-line values before stimulation. Differences between the stimulation by agonists with and without sulpiride cotreatment were analyzed by the SAS statistical package (SAS Institute, Cary NC) using the Student's *t* test.

[3 H]Dopamine uptake into striatal synaptosomes. Synaptosomal uptake followed the method of Steele *et al.* (1987) with minor modifications. Briefly, after dissection, pooled striata (six to eight animals) were homogenized immediately in 25 volumes of 0.32 M sucrose using a motor-driven Teflon-glass homogenizer (four gentle cycles). After centrifugation of the homogenate for 10 min at 1000 $\times$ g (4°C; Sorvall RC-5B/SS-34 rotor), the supernatant, representing the crude synaptosomes, was decanted and placed on ice until being added to the tubes. KB containing 118 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 0.03 mM Na₂EDTA, 0.6 mM ascorbic acid and 10 mM D-glucose (pH 7.4, oxygenated with 95% O₂/5% CO₂) was utilized throughout the assay, as well as for performing drug dilutions. Experimental conditions were run in duplicate, with each sample containing: 1.65 ml of KB, 0.2 ml of crude synaptosomes, 0.05 ml of pargyline (1 μ M), 0.05 ml of either buffer (control tubes) or drug, and 0.05 ml of [3 H]dopamine (10 nM) for a final volume of 2.0 ml. All components except the [3 H]dopamine were added to the tubes for a 5-min preincubation at 37°C. Radiolabeled dopamine was then added to all tubes for a 10-min incubation. This incubation was terminated by filtration through a Brandel cell harvester (Gaithersburg, MD) and rinsed with 15 ml of ice-cold KB. Filter mats were allowed to air dry, placed into scintillation vials and 4 ml of scintillation cocktail was added. Vials were shaken and radioactivity was quantified by liquid scintillation counting (LKB-1219 RackBeta). DHX was compared to GBR-12909, a selective dopamine uptake inhibitor, as well as to the endogenous ligand, dopamine. Nonspecific uptake was defined as that present at 4°C. Results are expressed as percent specific uptake (i.e., total minus nonspecific).

This method also was used for assessing the effects of DHX on the uptake of [3 H]serotonin into rat cortical synaptosomes. In these assays, DHX was compared to fluoxetine and serotonin.

Basal [3 H]Dopamine Release from Striatal Slices

The effects of DHX on the basal release of [3 H]dopamine from rat striatal slices was studied following the method, with minor modifications, of Booth *et al.* (1990). After dissection (two 1.2-mm coronal slices), the striata were cut into cubes of about 0.4 $\times$ 0.4 mm size with a McIlwain Tissue chopper. The slices were transferred to a test tube containing oxygenated KB (see [3 H]dopamine uptake for buffer constituents) and washed with 5 ml of buffer. Slices were resuspended (50 mg/ml) in Krebs' solution containing pargyline (10 μ M) and incubated for 5 min at 37°C. [3 H]Dopamine (ca. 100 nM) was added to the slices

and incubated for an additional 10 min. Slices were subsequently washed three times with fresh Krebs'. Approximately 5 mg of tissue in a 15- μ l volume were added to the reaction test tubes, which contained: 100 μ l of pargyline (100 μ M); 100 μ l of drug (or buffer); and 785 μ l of freshly oxygenated buffer. The reaction mixture was incubated at 37°C for 15 min with shaking and then placed on ice. The samples were centrifuged at 1700 $\times$ g for 10 min and 900 μ l of the supernatant were counted after the addition of 5 ml of Optiphase scintillation cocktail (LKB). Tissue slices were solubilized overnight in 1 ml of 1 N NaOH, and the radioactivity remaining in the slices was quantified using liquid scintillation spectrometry. Spontaneous "basal" release of [3 H]dopamine was defined as the percent of total radioactivity (supernatant plus tissue) in the supernatant, expressed as mean percent control $\pm$ S.E.M. Amphetamine served as the positive control for this series of release experiments.

Results

Affinity for dopamine receptors. The ability of DHX to compete for [3 H]SCH23390-labeled binding sites is shown in figure 2. This novel ligand competed for these radioligand binding sites in a concentration-dependent manner with an IC₅₀ value of approximately 10 nM. The dose-response curve of DHX is compared in figure 2 to those of the prototypic D₁ antagonist SCH23390 and the D₁ agonist SKF38393, as well as the D₂ antagonist domperidone. These receptor binding experiments show that DHX binds to D₁ receptors with a 3-fold greater affinity than the widely used D₁ agonist SKF38393.

Linear transformation of these binding data using indirect Hill plots provides a formal estimate of the IC₅₀ values and an indication [from the slope (n_H)] of the nature of the drug-receptor interaction. The n_H of DHX (like those of other agonists) was significantly less than one (ca. 0.7), whereas those of the antagonists were around unity (see under "Discussion"). Because the n_H values of the agonists were significantly less than one, all indices of affinity (except as noted in the following paragraph) are expressed as IC₅₀ rather than K_i values.

The D₁ receptor binding data of DHX were analyzed further based on a two-site model as the most parsimonious initial

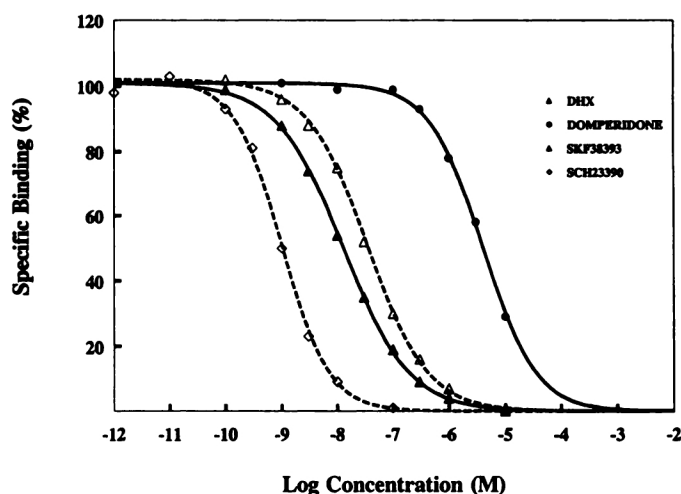


Fig. 2. DHX potently competes for [3 H]SCH23390-labeled D₁ receptors in rat striatal membranes. Representative curves showing the competition of DHX, SKF38393, SCH23390 and domperidone for [3 H]SCH23390-labeled binding sites. Data are plotted as percent specific binding (total – nonspecific), where nonspecific binding is defined by 1 μ M SCH23390. Each value represents the mean $\pm$ S.E.M. of data points, run in triplicate, from a single assay. For all data points, the S.E. bars fall within the size of the symbol.

choice. Since nonlinear regression of the data in the two-site model yielded an excellent fit ($r > 0.98$), no models of greater complexity were tested. The analysis was performed on the data from six assays performed on separate days, each utilizing between 9 and 15 concentration points per assay clustered about the IC_{50} . The data fit a two-site model with a high-affinity site ($K_i = 3.47 \pm 0.62$ nM; $63 \pm 5\%$ of total sites) and a low-affinity site ($K_i = 75 \pm 11$ nM; $37 \pm 5\%$ of total sites). Although such data could also be fitted by a model with three or more sites, there is no biological evidence at present to support use of a more complex model.

A summary of the IC_{50} values for DHX and related dopaminergic compounds at D₁ and D₂ receptors is shown in table 1. These results demonstrate that DHX displays a greater than 10-fold selectivity for D₁ receptors compared to D₂ receptors. This can be compared with the first reported full D₁ agonist, THP (Truex *et al.*, 1985; Andersen *et al.*, 1987), which has only 2-fold selectivity and more modest affinity for D₁ receptors, and SKF82958, a compound reported to be a full D₁ agonist

TABLE 1

Comparison of DHX and related compounds to compete for dopamine binding sites^a

Drug	D ₁ potency	D ₂ potency
	IC_{50} (nM) $\pm$ S.E.M.	
Dopamine	533 ± 43	72 ± 7
DHX	10 ± 1	122 ± 10
cis-DHX	$>5 \times 10^3$	$>5 \times 10^3$
N-methyl-DHX	91 ± 1	136 ± 29
N-propyl-DHX	651 ± 176	53 ± 4
N-allyl-DHX	328 ± 68	182 ± 36
BrMe-DHX	277 ± 44	$>3 \times 10^3$
THP	121 ± 32	285 ± 45
SKF38393	33 ± 2	$>3 \times 10^3$
SCH23390	1.0 ± 0.1	$>5 \times 10^3$
SKF82958	8 ± 2	145 ± 44
Ro 21-7767/001	955 ± 75	121 ± 4
Quinpirole	$>10^4$	98 ± 14
Domperidone	$>3 \times 10^3$	2.0 ± 1

^a D₁ potency was determined by competition binding for [³H]SCH23390 and D₂ potency by competition binding for [³H]spiperone in rat striatal membranes. n_H for the agonist binding curves were significantly less than 1; thus, K_i values cannot be determined until the number of binding sites is known. Some of the data in this table were taken from Brewster *et al.* (1990). The mean $IC_{50} \pm$ S.E.M. values represent data from 2 to 25 separate experiments.

TABLE 2

Comparison of DHX and related compounds on their ability to stimulate adenylate cyclase^{a,b}

Drug	Adenylate cyclase EC_{50}	Maximum Stimulation of Adenylate Cyclase
	nM	% vs. dopamine
Dopamine	5000	100
DHX	70	120
cis-DHX	$>10^4$	17
N-methyl-DHX	$>10^4$	67
N-propyl-DHX	$>10^4$	36
N-allyl-DHX	$>10^4$	32
BrMe-DHX	$>10^4$	12
THP	700	120
SKF38393	100^c	30–50
SCH23390	—	0
SKF82958	491	94
Ro 21-7767/001	$>10^4$	22

^a Activation of the D₁ receptor was assessed by determining the ability of the compound to activate adenylate cyclase in rat striatal membranes as described under "Methods."

^b Some of these data are abstracted from Brewster *et al.* (1990).

^c Concentration at which stimulation was one-half of the maximal value of 50%.

(O'Boyle *et al.*, 1989). Compared to DHX, SKF82958 has similar affinity for D₁ receptors and slightly greater selectivity for the D₁ subtype, but its pharmacology appears to be more "complex" (see under "Discussion"). These full agonists can be compared to the prototypal D₁ agonist SKF38393, which has greater D₁ vs. D₂ selectivity, but is only a partial efficacy D₁ agonist (see table 2). Table 1 also illustrates that *trans*-DHX (dihydroxidine) binds to D₁ receptors stereoselectively, as shown by the lack of affinity of the *cis*-isomer. It should be noted that although DHX displays a greater affinity for D₁ vs. D₂ receptors, it competes for [³H]spiperone binding sites with an IC_{50} of about 120 nM, an affinity similar to the widely used D₂ agonist quinpirole ($IC_{50} = 98$ nM).

N-alkylation of the nitrogen atom of DHX results in a diminution of D₁ affinity, but an increase in D₂ affinity. For example, methylation of the nitrogen moiety decreases the D₁ affinity approximately 9-fold, with little change in D₂ affinity. Increasing the alkylation chain length to a propyl group, however, results in dramatic loss of D₁ affinity and a corresponding 2- to 3-fold increase in D₂ affinity. Also noteworthy is the observation that N-propyl-DHX has greater D₂ receptor potency than the prototypic agonist quinpirole. In a fashion analogous to the N-allyl substituent of SKF82958, the N-allyl derivative of DHX was synthesized and found to have lower affinity for both D₁ and D₂ receptors. BrMe-DHX, the N-methyl-10-bromo-analog, with a substituent pattern similar to the D₁ antagonist SCH23390, had low D₁ receptor affinity. Ro 21-7767/001, another DHX "analog" synthesized previously by Hoffman-LaRoche, also was tested. The structure of this hexahydrobenzo[a]phenanthridine derivative is similar to DHX, except that it has 9,10-dihydroxy substituents and an additional N-methyl group. This compound had very little D₁ receptor affinity, but moderate D₂ receptor affinity.

Functional D₁ receptor activity: cAMP stimulation in homogenates. The characteristics of DHX in radioligand binding assays (*i.e.*, $n_H < 1$) suggested that it was a D₁ receptor agonist. This hypothesis was tested first by evaluating the ability of DHX to stimulate adenylate cyclase activity in rat striatal membranes. As reported previously (Lovenberg *et al.*, 1989; Brewster *et al.*, 1990), DHX stimulated adenylate cyclase activity to approximately the same degree as dopamine, thus showing that it is a full efficacy D₁ agonist (table 2). It is also noteworthy that DHX was 70-fold more potent than dopamine, as reflected by the EC_{50} values. The thienopyridine compound THP, as well as SKF82958, also stimulated adenylate cyclase activity fully, yet they were several-fold less potent than DHX. The prototypic D₁ agonist SKF38393 is only a partial agonist, as shown by its inability to activate the enzyme fully. As in radioreceptor assays, the *cis*-isomer of DHX was impotent and did not significantly stimulate adenylate cyclase activity. Methylation of the nitrogen atom of DHX resulted in a decreased efficacy and a loss of potency in this functional measure of D₁ activity. These effects were most pronounced with the N-propyl substitution, which caused a dramatic decrease in D₁ efficacy and potency (the latter shown by a 100-fold increase in the EC_{50} compared to DHX). N-allyl-DHX also was unable to stimulate adenylate cyclase activity fully. BrMe-DHX, a compound designed as a possible antagonist, displayed little activity at stimulating adenylate cyclase. Similar results were seen with Ro 21-7767, which was relatively inactive in the stimulation of adenylate cyclase.

To determine if the stimulation of adenylate cyclase activity

by DHX was solely the result of D_1 receptor activation, we tested the ability of various receptor antagonists to block DHX induction of cAMP synthesis. These antagonists and their targeted receptors were: SCH23390 (D_1 dopamine); atropine (muscarinic cholinergic); phenoxybenzamine (α -adrenergic); propranolol (β -adrenergic); ketanserin (5-hydroxytryptamine₂ serotonergic); and sulpiride (D_2 dopamine). As can be seen in figure 3, only the D_1 -selective antagonist SCH23390 was able to block the adenylate cyclase stimulation produced by 1 μ M dihydrexidine.

Functional D_2 receptor activity: Inhibition of prolactin release. Although the preceding data demonstrate that DHX is a high-potency, full efficacy D_1 agonist, table 1 indicates that it also competes for D_2 receptors in a manner suggestive of an agonist (i.e. $n_H < 1$). To test the hypothesis that DHX has D_2 agonist properties, we utilized an *in vivo* functional assay, which is based on the ability of D_2 agonists to inhibit prolactin release from pituitary lactotroph cells. In this paradigm, the animals were pretreated with the serotonin precursor 5-HTP to increase prolactin concentrations; this treatment increased the likelihood of detecting a D_2 agonist effect. As shown in figure 4, DHX inhibited significantly 5-HTP-induced prolactin release. Similarly, as previously reported, the D_2 agonist quinpirole also produced a similar inhibition. These results provide evidence in support of the hypothesis that the D_2 properties of DHX are probably agonistic in nature.

cAMP efflux from striatal slices demonstrates functional D_1 and D_2 effects of DHX. Superfused striatal slices were used to estimate the nature of the D_2 receptor actions of DHX. As in homogenates or single cells (Lovenberg *et al.*, 1991), DHX was fully efficacious (relative to dopamine) in stimulating cAMP efflux from superfused striatal slices. This assay of adenylate cyclase activation was chosen because previous studies (Stoof and Kebabian, 1981; Cook *et al.*, 1991) have shown that it demonstrates not only stimulatory effects of D_1 receptor activation, but also the inhibitory influence of

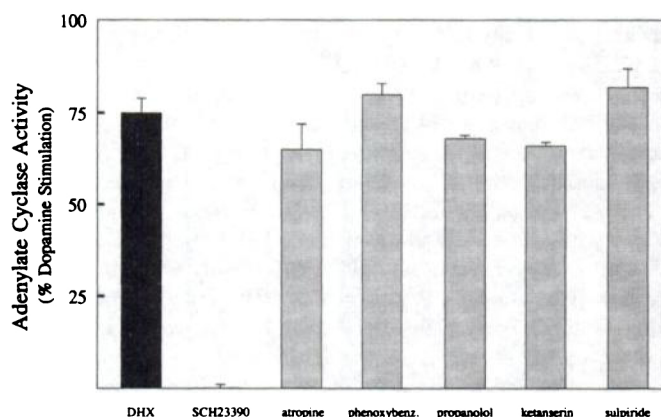


Fig. 3. The stimulation of adenylate cyclase by DHX can only be blocked by the D_1 antagonist SCH23390. The ability of various receptor antagonists to block the stimulation of adenylate cyclase activity in rat striatal membranes by 1 μ M DHX. The ■ represents stimulation produced by DHX alone, whereas ■ indicate DHX plus an antagonist. The method used to determine the antagonist effects is the same as that listed in table 2. Results are expressed as adenylate cyclase stimulation (% dopamine) where the stimulation produced by 100 μ M dopamine is equal to 100%. The receptor antagonists and the concentration used are the following: SCH23390 (1 μ M); atropine (10 μ M); phenoxybenzamine (10 μ M); propranolol (10 μ M); ketanserin (1 μ M); and sulpiride (1 μ M). Error bars represent the S.E.M. for two to eight separate experiments.

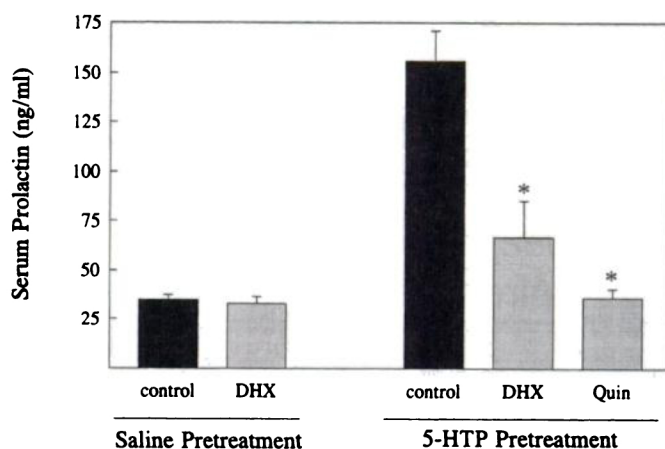


Fig. 4. DHX and quinpirole inhibit 5-HTP-induced prolactin release. To assess the ability of DHX to activate pituitary D_2 receptors, thereby inhibiting prolactin release, 5-HTP was used to increase prolactin secretion. Both DHX and the D_2 agonist, quinpirole, caused statistically significant inhibition of prolactin release. Values for 5-HTP pretreatment represent the mean $\pm$ S.E.M. of four animals per condition, whereas the saline pretreatment groups represent the mean of two animals. *, Statistically significant difference from 5-HTP alone ($P < .05$).

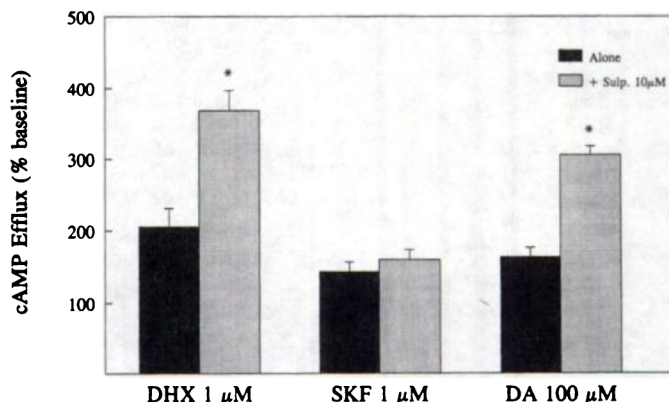


Fig. 5. cAMP efflux assays reveal both D_1 and D_2 functional properties of DHX. DHX causes increases in cAMP efflux from striatal slices. This is blocked by SCH23390 (data not shown). The cAMP efflux caused by DHX can be increased further by the presence of the D_2 antagonist sulpiride (10 μ M), suggesting that at higher doses, DHX has D_2 agonist properties that can be blocked by sulpiride. A similar effect is shown for dopamine, but not for SKF38393, a partial but selective D_1 agonist. The results represent the mean of four wells from two separate experiments. *, Signifies statistically significant ($P < .05$) effect of sulpiride vs. respective agonist.

D_2 receptor stimulation, an effect not detectable in striatal homogenates (unpublished observations). Figure 5 shows that DHX has a functional D_2 component in affecting stimulation of cAMP efflux. These data suggest that cAMP efflux produced by 1 μ M DHX represents a balance between stimulation of D_1 receptors and a D_2 receptor-mediated inhibition of cAMP synthesis. Thus, cotreatment with the D_2 receptor antagonist sulpiride resulted in a blockade of DHX- D_2 receptor-mediated inhibition and produced an enhanced cAMP efflux. This D_2 receptor component to cAMP efflux is also seen with dopamine, but not with the selective D_1 receptor agonist SKF38393. These data are consistent with the prolactin inhibition experiments above, showing that DHX also possesses D_2 receptor agonist properties.

Affinity for other neurotransmitter receptors. Having previously determined that DHX is very active at dopamine

receptors, it was essential to evaluate its overall selectivity. DHX was screened with 31 different neurotransmitter radioligands, as well as against eight other binding sites associated with cell signaling. These data (table 3) demonstrate that DHX has little affinity for nondopamine receptor sites. In this receptor screening, the most potent DHX response was with α_2 adrenergic receptors. In a more detailed examination, DHX competed for α_2 adrenoceptor binding sites labeled with [³H]atipamizole (IC_{50} = ca. 230 nM) more potently than was determined from the screening assays, which used [³H]RX781094 (IC_{50} = ca. 1 μ M) as the radioligand. The competition data shown in table 3 for [³H]ketanserin or [³H]prazosin binding sites is essentially identical to those data generated in this laboratory. Together, these results demonstrate that dihydropyridine is, with the exception of weak α_2 adrenergic receptor activity, highly selective for dopamine receptors.

Affinity for the dopamine uptake site. The pharmacological properties of DHX were investigated further to determine whether any of its activity results from the interaction at the dopamine presynaptic uptake site. DHX only weakly inhibited (IC_{50} > 10 μ M) the uptake of radiolabeled dopamine into

rat striatal synaptosomes (fig. 6). The response of DHX was compared to the selective dopamine uptake inhibitor GBR-12909 (IC_{50} ca. 24 nM) and the natural ligand dopamine (IC_{50} ca. 366 nM). These data indicate that the effects produced by DHX are not the result of its interaction at the dopamine uptake site. In addition, we assessed the ability of DHX to compete for the serotonin uptake site. The results from these experiments demonstrated that DHX only weakly inhibited uptake (IC_{50} > 10 μ M), whereas fluoxetine (IC_{50} ca. 47 nM) and serotonin (IC_{50} ca. 55 nM) potently inhibited the uptake of [³H]serotonin into cortical synaptosomes (data not shown).

Effects of basal [³H]dopamine release. In further experiments, we assessed whether the pharmacological properties of DHX also could involve "indirect" effects at dopamine nerve terminals. More specifically, we determined whether DHX, like amphetamine, could stimulate the release of [³H]dopamine from preloaded rat striatal slices. Utilizing this technique, DHX only weakly altered the basal release of [³H]dopamine (fig. 7). The magnitude of this weak response was similar to that of the direct acting agonists quinpirole (D₂ receptor) and SKF38393 (D₁ receptor), but in sharp contrast to the marked response

TABLE 3
Affinity of DHX for other receptor sites*

Receptor	Selectivity	Radioligand Used to Label Binding Sites	IC ₅₀ of DHX μ M
Adenosine	Adenosine	[³ H]NECA	>10
Adrenergic	α_1	[³ H]Prazosin	>10
	α_2	[³ H]RX781094	>1
	α_2	[³ H]Atipamizole	0.23 ^b
	β	[³ H]Iodopindolol	>10
		[³ H]Glycine	>10
Excitatory amino acids	Kainate	[³ H]Kainic acid	>10
	MK-801	[³ H]MK-801	>10
	NMDA	[³ H]CGS 19755	>10
	PCP	[³ H]TCP	>10
	Quisqualate	[³ H]AMPA	>10
	Sigma	[³ H]DTG	>10
	Benzodiazepine	[³ H]Flunitrazepam	>10
	GABA _A	[³ H]GABA	>10
Inhibitory amino acids	GABA _B	[³ H]GABA + Isoguvacine*	>10
	Glycine	[³ H]Strychnine	>10
	Serotonin 1	[³ H]5-HT	>10
	Serotonin 2	[³ H]Ketanserin	=10
Serotonergic	Interleukin	[¹²⁵ I]IL 1- α	>10
Biological response modifiers	Calcium	[³ H]Nitrendipine	>10
	Calcium	[¹²⁵ I]- ω -conotoxin	>10
	Chloride	[³ H]TBOB	>10
	Potassium	[¹²⁵ I]Apamin	>10
	ANF	[¹²⁵ I]ANP	>10
Peptide Factors	EGF	[¹²⁵ I]EGF	>10
	NGF	[¹²⁵ I]NGF	>10
	Angiotensin	[¹²⁵ I]Angiotensin II	>10
Peptides	Arg-vasopressin V1	[³ H]Manning compound	>10
	Bombesin	[¹²⁵ I]GRP	>10
	CCK central	[¹²⁵ I]CCK	>10
	CCK peripheral	[¹²⁵ I]CCK	>10
	Neurotensin	[³ H]Neurotensin	>10
	NPY	[¹²⁵ I]NPY	>10
	Somatostatin	[¹²⁵ I]Somatostatin	>10
	Substance K	[¹²⁵ I]Neurokinin A	>10
	Substance P	[³ H]Substance P	>10
	VIP	[¹²⁵ I]VIP	>10
	Forskolin	[³ H]Forskolin	>10
	Phorbol ester	[³ H]PDBU	>10
Second messenger systems:	Inositol triphosphate	[³ H]IP ₃	>10
Adenylate cyclase			
Protein kinase C			

* See under "Methods" for details of the screening of DHX in these assays.

^b This value represents a full competition curve run using [³H]atipamizole.

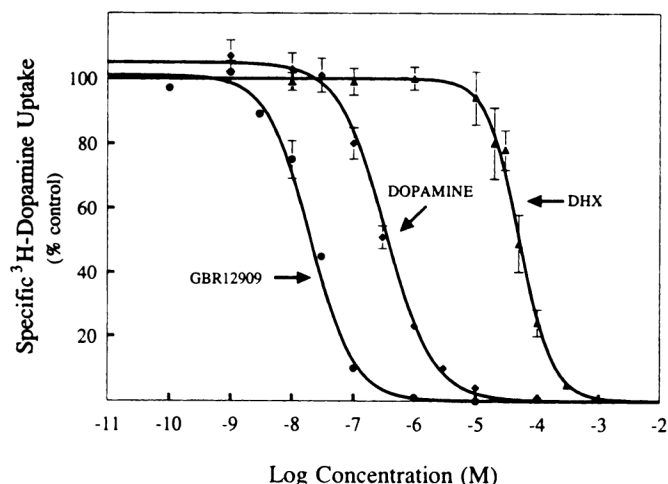


Fig. 6. DHX weakly competes for the dopamine uptake site. The relative potency of DHX, dopamine and GBR-12909 for inhibiting the uptake of [3 H]dopamine into rat striatal synaptosomes is shown. Results are expressed as specific [3 H]dopamine uptake (% control), where control values represent the difference between uptake at 37°C and at 4°C. With the exception of 3 and 30 nM GBR-12909, each value represents the mean $\pm$ S.E.M. of two to eight separate experiments. For some data points, the S.E. bars fall within the size of the symbol.

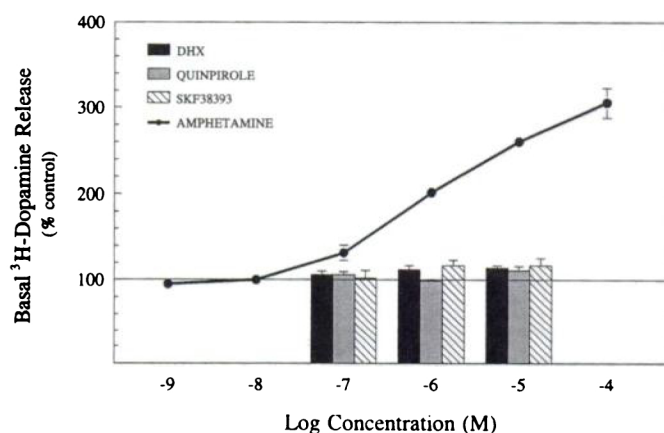


Fig. 7. DHX does not induce amphetamine-like dopamine release. DHX only produced small alterations in [3 H]dopamine release in prelabeled striatal slices. This DHX effect (■) is similar to the D_2 agonist quinpirole (▨) and the D_1 agonist SKF38393 (▤). Conversely, as expected, amphetamine (line) caused a profound dose-related release of dopamine. Each data point represents the mean $\pm$ S.E.M. of at least three separate experiments. For some amphetamine data points, the S.E. bars fall within the size of the symbol.

caused by amphetamine. This series of experiments suggests that the functional properties of DHX are not the result of DHX-induced dopamine release from dopaminergic terminals.

Discussion

Although the availability of selective D_1 antagonists like SCH23390 has been critical in elucidating mechanisms of D_1 receptor function, there have not been appropriate full efficacy agonists available to complement such studies. The endogenous ligand dopamine displays relatively low potency and selectivity at D_1 -like receptors, and, although the substituted phenyl-tetrahydrobenzazepines (e.g., SKF38393) are of high potency, they are only partially efficacious relative to dopamine. Thus, full efficacy, high-potency compounds like DHX are of great

potential utility. The present study provides pharmacological and functional data relevant toward such uses of DHX.

As was shown in initial reports (Lovenberg *et al.*, 1989; Brewster *et al.*, 1990), DHX competes for [3 H]SCH23390-labeled D_1 receptors with high affinity, approximately 3-fold greater than the widely used D_1 receptor agonist SKF38393. Like SKF38393, DHX displays "shallow" competition curves, resulting in an indirect n_H of approximately 0.7. These "complex" kinetics are suggestive of either different affinity states of one receptor or different molecular forms of the D_1 -like receptor. Such behavior is seen commonly with other members of the G-protein receptor superfamily. Nonlinear regression of the data in these DHX competition curves was resolved by a two-site model. The model estimated a high-affinity site of $K_D = 3$ nM (63% of the sites) and a low-affinity site of $K_D = 75$ nM (37%). Under the binding conditions used, the addition of guanine nucleotides caused a parallel shift to the right in the competition curve (i.e., no change in n_H). Because the addition of GTP analogs to shallow agonist binding curves typically produces a rightward shift and steepening of the curve for other receptor systems (e.g., β adrenergic), a number of experimental modifications were attempted to try to induce a steepening of the competition curve (i.e., change n_H to ca. 1). A change in n_H to approximately 1 would suggest a guanine nucleotide-induced conversion of a high-affinity receptor state to a low-affinity state. None of the experimental manipulations used (different GTP analogs; ion composition; fresh *vs.* frozen tissue; different buffers) qualitatively affected the results. Previous reports have demonstrated interlaboratory differences in guanine nucleotide effects on D_1 receptors (Richfield *et al.*, 1989; Hess *et al.*, 1986; Niznik *et al.*, 1986), suggesting that many factors, or the interaction of several, may affect the receptor affinity state and resulting guanine nucleotide sensitivity. The present data suggest that the majority of DHX-sensitive receptors in our preparations are high-affinity sites.

One biochemical characteristic that distinguishes DHX from the phenyltetrahydrobenzazepines is its full efficacy in the activation of D_1 receptors, at least with respect to the generally accepted biochemical endpoint, cAMP synthesis. As reported earlier, DHX was equal to dopamine and more than twice as efficacious as SKF38393 in stimulating cAMP synthesis in rat striatum (Lovenberg *et al.*, 1989; Brewster *et al.*, 1990) and in NS20Y neuroblastoma cells (Lovenberg *et al.*, 1991). Consistent with the radioreceptor studies, the potency of DHX in activating D_1 receptors was significantly greater than these other agents. One report (O'Boyle *et al.*, 1989) described SKF82958, another phenyltetrahydrobenzazepine, as a D_1 agonist with significantly greater than full activation, producing a 50% greater increase in cAMP synthesis than did dopamine, but with relatively low affinity ($K_i = \text{ca. } 330$ nM; O'Boyle and Waddington, 1987).

In our hands, however, SKF82958 was approximately equal in potency to DHX in D_1 radioreceptor assays. Moreover, SKF82958 appeared to activate adenylate cyclase fully, although the dose-response curve was extremely shallow (greater than 5 log orders of concentration from partial to full response), ultimately requiring approximately 100 μ M to obtain full activation. Although the explanation of this shallow and atypical dose-response curve will require additional studies, preliminary results indicate that these effects of SKF82958 can be blocked by SCH23390 (1 μ M), suggesting a complex mechanism(s) involving D_1 receptors. One explanation for these complex

interactions of SKF82958 is that this compound acts by two distinct mechanisms (*e.g.*, providing two pharmacophores to the D₁ receptor or binding to two "D₁-like" receptors). Although further experiments are necessary to clarify these issues, it is clear that DHX does not suffer from the same limitations or complications.

In our preliminary report about DHX (Lovenberg *et al.*, 1989), we demonstrated that this drug exhibited affinity for both D₁ and D₂ dopamine receptors, but we did not address the possible interaction with other receptor systems. The present data describe a comprehensive screening of the interaction of DHX with more than three dozen other receptor types. DHX displayed at least 100-fold selectivity for dopamine receptors compared to other receptor types, with the sole exception of the α_2 adrenergic receptors. A more detailed characterization of the α_2 adrenoreceptor potency was conducted using [³H]atipamizole as a radioligand. Under our assay conditions, DHX competed for the radiolabeled α_2 sites with an IC₅₀ of about 230 nM and a Hill slope of about 0.7. These data suggest that DHX may be a weak agonist for α_2 receptors; this may necessitate appropriate experimental controls in experiments using high concentrations of DHX, or when DHX is used with tissue preparations containing a high density of α_2 adrenoreceptors (*e.g.*, olfactory nucleus, cingulate cortex).

A more important factor in the pharmacology of DHX relates to its potency at D₂ receptors (see table 1). As reported previously and confirmed in aspects of the present work, DHX has an order of magnitude selectivity for D₁ vs. D₂ receptors (Lovenberg *et al.*, 1989; Brewster *et al.*, 1990). As we have noted, however, these D₂ properties make DHX similar in potency to the prototypical D₂ agonist quinpirole (table 1). Two lines of studies were performed to determine whether the D₂ properties of DHX reflected agonist or antagonist properties. The first used inhibition of prolactin secretion, a well-documented D₂-mediated effect that is not markedly affected by α_2 adrenoreceptors. As expected, rats pretreated with 5-HTP had the expected increase in serum prolactin concentrations. As was shown in figure 4, DHX caused significant decrease in 5-HTP-induced prolactin secretion, thus demonstrating that DHX possesses D₂ agonist properties.

Another line of evidence that the D₂ properties of DHX were agonistic in nature came from studies of cAMP efflux using rat striatal slices. In slices, as well as in single cell and molecular expression systems, but not striatal homogenates, agonist occupation of D₂ receptors results in inhibition of D₁-stimulated cAMP synthesis. As was shown in figure 5, DHX, SKF38393 and dopamine increased cAMP efflux. Addition of the D₂ antagonist sulpiride produced a further increase of the cAMP synthesis induced by DHX and dopamine, but not by SKF38393. These data are consistent with the fact that in the striatum, sulpiride blocks the inhibitory D₂ agonist effects of DHX or dopamine, but not of SKF38393, an agonist of known D₁ selectivity. These data provide a second line of evidence that DHX acts as a D₂, as well as a D₁ receptor agonist.

As a catecholamine, it is possible that some of these functional effects of DHX are not direct receptor-mediated events, but may result from increases in synaptic concentrations of dopamine *via* inhibition of uptake or release of dopamine. These possibilities were tested directly using several preparations. DHX was found to compete only weakly for the dopamine uptake site (IC₅₀ > 10 μ M; fig. 5). Moreover, DHX did not

stimulate the release of dopamine as does amphetamine (fig. 6). Together, these data suggest that the functional dopaminergic properties of DHX are due to direct receptor-coupled events rather than actions as an indirect agonist.

Because DHX possessed ideal D₁ receptor pharmacological characteristics and represented a novel structural class of dopaminergic ligands, structure-activity relationship (SAR) analysis of this class was undertaken to aid our description of the D₁ and D₂ receptor pharmacophore. Modifications to the structure of DHX (see fig. 1) resulted in an interesting pattern of changes in pharmacological activity. The addition of a methyl group to the nitrogen atom of DHX resulted in a decrease in D₁, but not D₂, receptor affinity, as well as a corresponding decrease in D₁ efficacy. The addition of a propyl group to the nitrogen atom of DHX resulted in a loss of D₁ receptor activity, but greatly enhanced potency at D₂ receptors. In fact, the N-propyl derivative was twice as potent as the prototypic D₂ agonist quinpirole. It thus appears that with the proper structural modifications, the benzo[a]phenanthridine class also can generate potent and selective D₂ ligands. These SAR data show that the D₁ receptor is sensitive to alkyl substitution at the benzo[a]phenanthridine nitrogen atom, whereas the D₂ receptor may better accommodate such additions. The weak activity of N-allyl-DHX and BrMe-DHX demonstrate that chemical group substitution patterns similar to those used to enhance activity of the tetrahydrobenzazepine class do not result in a corresponding increase in activity in this novel class. In addition, the decreased activity of Ro 21-7767 shows that the positioning of the hydroxyl groups is critical for D₁ activation. Thus, the hexahydrobenzo[a]phenanthridine class will allow further exploration of dopamine receptor recognition characteristics.

An exciting outgrowth of these data will require the separation of the enantiomers of DHX. All studies described here utilized DHX as a mixture of two enantiomers (6aR,12bS and 6aS,12bR). Therefore, the competition between the stereoisomers may underestimate the potency of the active enantiomer. Based on molecular modeling studies, we have hypothesized that 6aR,12bS-DHX will be the more active D₁ ligand (Mottola *et al.*, 1990; submitted, Brewster *et al.*, 1990). The resolution of the enantiomers may result in a more selective, higher potency agonist.

The present studies provide a mechanistic basis for understanding the unique psychopharmacological effects of DHX in rats (Darney *et al.*, 1991) and in normal monkeys (Elsworth *et al.*, 1991). In addition to its utility as a pharmacological tool to study D₁ receptor function, we have proposed previously (Lovenberg *et al.*, 1989; Brewster *et al.*, 1990) that a full efficacy D₁ agonist like DHX may have special clinical utility as an antiparkinsonian drug. Recently, we have reported that DHX produced dramatic improvements in parkinsonian signs in severely affected MPTP-treated African Green monkeys (Taylor *et al.*, 1991). One of the interesting questions raised by the present work is whether the D₂ properties of DHX, now shown to be agonistic in nature, are important in its antiparkinsonian actions in primates. It remains to be resolved whether it is solely the D₁ actions of DHX or a combination of its D₁ activity coupled with D₂ activation (from DHX and/or residual dopamine neurons) that are required for antiparkinsonian effects. Further characterization of the nature of the D₂ properties of DHX and its N-propyl derivative now seems appropriate, especially because DHX shows great promise as a novel thera-

peutic agent to treat advanced parkinsonism (Taylor *et al.*, 1991).

In summary, DHX represents the most active and efficacious D₁ dopaminergic member of the hexahydrobenzo[a]phenanthridine series. Although this ligand also possesses reasonable D₂ affinity, it is highly selective for dopamine receptors. The present data demonstrate that the pharmacological effects of this ligand (Darney *et al.*, 1991; Taylor *et al.*, 1991; Elsworth *et al.*, 1991) are due to direct receptor activation rather than indirect effects at either the dopamine uptake site or in the release of dopamine from the nerve terminal. Together, these results support the contention that DHX (and the benzo[a]phenanthridine class) will be instrumental in resolving the determinants of ligand recognition by dopamine receptor, in elucidating D₁ receptor function and in probing D₁/D₂ receptor interactions.

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References

- ANDERSEN, P. H., NIELSEN, E. B., SCHEEL-KRUGER, J., JANSEN, J. A. AND HOHLWEG, R.: Thienopyridine derivatives identified as the first selective, full efficacy, dopamine D₁ receptor agonists. *Eur. J. Pharmacol.* **137**: 291-292, 1987.
- BISHOP, J. E., MATHIS, C. A., GERDES, J. M., WHITNEY, J. M., EATON, A. AND MAILMAN, R. B.: Synthesis and in vitro evaluation of 2,3-dimethoxy-5-fluoroalkyl-substituted benzamides and salicylamides: High affinity ligands for CNS dopamine D₂ receptors. *J. Med. Chem.* **34**: 1612-1624, 1991.
- BOOTH, R. G., BALDESSARINI, R. J., KULA, N. S., GAO, Y., ZONG, R., NEUMEYER, J. L.: Presynaptic inhibition of dopamine synthesis in rat striatal tissue by enantiomeric mono- and dihydroxyaporphines. *Mol. Pharmacol.* **38**: 92-101, 1990.
- BREWSTER, W. K.: Studies directed toward the synthesis of certain trans-fused benzo[a]phenanthridines as structurally rigid dopamine D-1 agonists and antagonists (Dissertation). Purdue University, 1990.
- BREWSTER, W. K., NICHOLS, D. E., RIGGS, R. M., MOTTOLA, D. M., LOVENBERG, T. W., LEWIS, M. H. AND MAILMAN, R. B.: *Trans*-10,11-dihydroxy-5,6,6a,7,8,12b-hexahydrobenzo[a]phenanthridine: A highly potent selective dopamine D₁ full agonist. *J. Med. Chem.* **33**: 1756-1764, 1990.
- BUNZOW, J. R., VAN TOL, H. H. M., GRANDY, D. K., ALBERT, P., SALON, J., CHRISTIE, M., MACHIDA, C. A., NEVE, K. A. AND CIVELLI, O.: Cloning and expression of a rat D₂ dopamine receptor cDNA. *Nature* **336**: 783-787, 1988.
- CHARIFSON, P. S., BOWEN, J. P., WYRICK, S. D., HOFFMAN, A. J., CORY, M., MCPHAIL, A. T. AND MAILMAN, R. B.: Conformational analysis and molecular modeling of 1-phenyl-, 4-phenyl-, and 1-benzyl-1,2,3,4-tetrahydroisoquinolines as D₁ dopamine receptor ligands. *J. Med. Chem.* **32**: 2050-2058, 1989.
- CHRISTENSEN, A. V., ARNT, J., HYTTTEL, J., LARSEN, J. J. AND SVENDSEN, O.: Pharmacological effects of a specific dopamine D-1 antagonist SCH23390 in comparison with neuroleptics. *Life Sci.* **34**: 1529-1540, 1984.
- CLARK, D. AND WHITE, F. J.: Review: D₁ dopamine receptor—The search for a function: A critical evaluation of the D₁/D₂ dopamine receptor classification and its functional implications. *Synapse* **1**: 347-388, 1987.
- COOK, L. L., MOTTOLA, D. M., PETTITTO, J. M., LEWIS, M. H. AND MAILMAN, R. B.: Functional interactions of striatal D₁ and D₂ dopamine receptors: Effects of tissue preparation and lesions. *Soc. Neurosci. Abstr.* **17**: 241.6, 1991.
- DARNEY, K. J., LEWIS, M. H., BREWSTER, W. K., NICHOLS, D. E. AND MAILMAN, R. B.: Behavioral effects in the rat of dihydroxidine, a high potency, full efficacy D₁ dopamine receptor agonist. *Neuropsychopharmacology* **5**: 187-195, 1991.
- DEARRY, A., GINGRICH, J. A., FALARDEAU, P., FREMEAUX, R. T., BATES, M. D. AND CARON, M. G.: Molecular cloning and expression of the gene for a human D₁ dopamine receptor. *Nature* **347**: 72-76, 1990.
- EIDNE, K., TAYLOR, P. L., ZABAVNIK, J., SAUNDERS, P. T. K. AND INGLIS, J. D.: D₂ receptor, a missing exon. *Nature* **342**: 865, 1989.
- ELSWORTH, J. D., LAWRENCE, M. S., ROTH, R. H., TAYLOR, J. R., MAILMAN, R. B., NICHOLS, D. E., LEWIS, M. H. AND REDMOND JR., D. E.: D₁ and D₂ Dopamine receptors independently regulate spontaneous blink rate in the Vervet monkey. *J. Pharmacol. Exp. Ther.* **259**: 595-600, 1991.
- GINGRICH, J. A., DEARRY, A. C., FALARDEAU, P., FREMEAUX, R. T., BATES, M. D. AND CARON, M. G.: Molecular characterization of G-protein coupled receptors: Isolation and cloning of a D₁ dopamine receptor. *J. Recept. Res.* **11**: 521-534, 1991.
- GIROS, B., SOKOLOFF, P., MARTRES, M. P., RIOU, J. F., EMORINE, L. J. AND SCHWARTZ, J. C.: Alternative splicing directs the expression of two D₂ dopamine receptor isoforms. *Nature* **342**: 923-926, 1989.
- HARPER, J. F. AND BROOKER, G.: Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'-O-acetylation by acetic anhydride in aqueous solution. *J. Cyclic Nucleotide Res.* **1**: 207-218, 1975.
- HEFFNER, T. G., HARTMAN, J. A. AND SEIDEN, L. S.: A rapid method for the regional dissection of the rat brain. *Pharmacol. Biochem. Behav.* **13**: 453-456, 1980.
- HERR, D. W. AND MAILMAN, R. B.: Buffer effects on high affinity [3H]-prazosin binding in brain and spinal cord. *Pharmacol. Biochem. Behav.* **32**: 831-834, 1989.
- HESS, E. J., BATTAGLIA, G., NORMAN, A. B., IORIO, L. C. AND CREESE, I.: Guanine nucleotide regulation of agonist interactions at [3H]SCH23390-labeled D₁ dopamine receptors in rat striatum. *Eur. J. Pharmacol.* **121**: 31-38, 1986.
- IORIO, L. C., BARNETT, A., LEITZ, F. H., HOUSER, V. P. AND KORDUBA, C. A.: SCH23390, a potential benzazepine antipsychotic with unique interactions on dopaminergic systems. *J. Pharmacol. Exp. Ther.* **226**: 462-468, 1983.
- KEBABI, J. W. AND CALNE, D. B.: Multiple receptors for dopamine. *Nature* **277**: 93-96, 1979.
- KUHN, C. M., VOGEL, R. A., MAILMAN, R. B., MUELLER, R. A., SCHANBERG, S. M. AND BREESE, G. R.: Effects of 5,7-dihydroxytryptamine on serotonergic control of prolactin secretion and behavior in rats. *Psychopharmacology* **73**: 188-193, 1981.
- LOVENBERG, T. W., BREWSTER, W. K., MOTTOLA, D. M., LEE, R. C., RIGGS, R. M., NICHOLS, D. E., LEWIS, M. H. AND MAILMAN, R. B.: Dihydroxidine, a novel selective high potency full dopamine D₁ receptor agonist. *Eur. J. Pharmacol.* **166**: 111-113, 1989.
- LOVENBERG, T. W., NESTLER, E. J., ROTH, R. H., NICHOLS, D. E. AND MAILMAN, R. B.: Guanine nucleotide binding proteins and the regulation of cyclic AMP synthesis in NS20Y neuroblastoma cells: Role of D₁ dopamine and muscarinic receptors. *Brain Res.* **556**: 101-107, 1991.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. AND RANDALL, R. J.: Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**: 265-275, 1951.
- MAILMAN, R. B., SCHULZ, D. W., KILTS, C. D., LEWIS, M. H., ROLLEMA, H. AND WYRICK, S.: Multiplicity of the D₁ dopamine receptor. In *Neurobiology of Central D₁ Receptors*, ed. by G. R. Breeze and I. Creese, pp. 53-72, Plenum Press, New York 1986.
- MAILMAN, R. B., SCHULZ, D. W., LEWIS, M. H., STAPLES, L., ROLLEMA, H. AND DEHAVEN, D. L.: SCH23390: A selective D₁ dopamine receptor antagonist with potent D₂ behavioral actions. *Eur. J. Pharmacol.* **101**: 159-160, 1984.
- MCPHERSON, G. A.: A practical computer based approach to the analysis of radioligand binding experiments. *Comput. Programs Biomed.* **17**: 107-114, 1983.
- MONSMA, F. J., MAHAN, L. C., MCVITTIE, L. D., GERFEN, C. R. AND SIBLEY, D. R.: Molecular cloning and expression of a D₁ dopamine receptor linked to adenylate cyclase activation. *Proc. Natl. Acad. Sci. USA* **87**: 6273-6277, 1990.
- MONSMA, F. J., MCVITTIE, L. D., GERFEN, C. R., MAHAN, L. C. AND SIBLEY, D. R.: Multiple D₁ dopamine receptors produced by alternative RNA splicing. *Nature* **342**: 926-929, 1989.
- MOTTOLA, D. M., BREWSTER, W. K., NICHOLS, D. E. AND MAILMAN, R. B.: Dihydroxidine, a novel agonist to study the pharmacology and structural requirements of D₁ dopamine receptors. *Soc. Neurosci. Abstr.* **16**: 37.4, 1990.
- NICHOLS, D. E.: The development of novel dopamine agonists. In *ACS Symposium series No. 224*, ed. by C. Kaiser and J. W. Kebabian, pp. 201-218, Am. Chem. Soc., Washington, D.C., 1983.
- NIZNIK, H. B., OTSUKA, N. Y., DUMBRILLE-ROSS, A., TIRPAK, A. AND SEEMAN, P.: Dopamine D₁ receptors characterized with [3H]SCH23390. *J. Biol. Chem.* **261**: 8397-8406, 1986.
- O'BOYLE, K. M., GAITANOPOULOS, D. E., BRENNER, M. AND WADDINGTON, J. L.: Agonist and antagonist properties of benzazepine and thienopyridine derivatives at the D₁ dopamine receptor. *Neuropharmacology* **28**(4): 401-406, 1989.
- O'BOYLE, K. M. AND WADDINGTON, J. L.: New substituted 1-phenyl-3-benzazepine analogues of SKF 38393 and N-methyl-thienopyridine analogues of dihydroxynomifensine with selective affinity for the D₁ dopamine receptor in human post-mortem brain. *Neuropharmacology* **26**(12): 1807-1810, 1987.
- PATEL, A. AND LINDEN, J.: Purification of ¹²⁵I-labelled succinyl cyclic nucleotide tyrosine methyl esters by high-performance liquid chromatography. *Anal. Biochem.* **168**: 417-420, 1988.
- RICHFIELD, E. K., PENNEY, J. B. AND YOUNG, A. B.: Anatomical and affinity state comparisons between dopamine D₁ and D₂ receptors in the rat central nervous system. *Neuroscience* **30**: 767-777, 1989.
- SCHULZ, D. W. AND MAILMAN, R. B.: An improved, automated adenylate cyclase assay utilizing preparative HPLC: effects of phosphodiesterase inhibitors. *J. Neurochem.* **42**: 764-774, 1984.
- SCHULZ, D. W., STANFORD, E. J., WYRICK, S. W. AND MAILMAN, R. B.: Binding of [3H]-SCH23390 in rat brain: Regional distribution and effects of assay condition and GTP suggest interactions at a D₁-like dopamine receptor. *J. Neurochem.* **45**: 1601-1611, 1985.
- SOKOLOFF, P., GIROS, B., MARTRES, M. P., BOUTHENET, M. L. AND SCHWARTZ, J. C.: Molecular cloning and characterization of a novel dopamine receptor (D₃) as a target for neuroleptics. *Nature* **347**: 146-151, 1990.
- STEELE, T. D., NICHOLS, D. E. AND YIM, G. K. W.: Stereochemical effects of 3,4-methylenedioxymethamphetamine (MDMA) and related amphetamine derivatives on inhibition of uptake of [3H]monoamines into synaptosomes from different regions of rat brain. *Biochem. Pharmacol.* **36**: 2297-2303, 1987.
- STOFF, J. C. AND KEBABI, J. W.: Opposing roles for D-1 and D-2 dopamine receptors in efflux of cyclic AMP from rat neostriatum. *Nature* **294**: 366-368, 1981.

- SUNAHARA, R. K., GUAN, H. C., O'DOWD, B. F., SEEMAN, P., LAURIER, L. G., NG, G., GEORGE, S. R., TORCHIA, J., VAN TOL, H. H. M. AND NIZNIK, H. B.: Cloning of the gene for a human dopamine D₂ receptor with high affinity for dopamine than D₁. *Nature* **250**: 614-618, 1991.
- SUNAHARA, R. K., NIZNIK, H. B., WEINER, D. M., STORMANN, T. M., BRANN, M. R., KENNEDY, J. L., GELERNTER, J. E., ROZMAHEL, R., YANG, Y., ISREAL, Y., SEEMAN, P. AND O'DOWD, B. F.: Human dopamine D₁ receptor encoded by intronless gene on chromosome 5. *Nature* **347**: 80-83, 1990.
- TAYLOR, J. R., LAWRENCE, M. S., REDMOND JR., D. E., ELSWORTH, J. D., ROTH, R. H., NICHOLS, D. E. AND MAILMAN, R. B.: Dihydropyridine, a full dopamine D₁ agonist, reduces MPTP-induced parkinsonism in African green monkeys. *Eur. J. Pharmacol.* **199**: 387-388, 1991.
- TIBERI, M., JARVIE, K. R., SILVIA, C., FALARDEAU, P., GINGRICH, J. A., GODINOT, N., BERTRAND, L., YANG-FENG, T. L., FREMEAU, R. T. AND CARON, M.: Cloning, molecular characterization, and chromosomal assignment of a gene encoding a second D₁ dopamine receptor subtype: Differential expression pattern in rat brain compared with the D₁ receptor. *Proc. Natl. Acad. Sci. USA* **88**: 7491-7495, 1991.
- TRUEX, L. L., FOREMAN, M. M., RIGGS, R. M. AND NICHOLS, D. E.: Effects of modifications of the 4-(3,4-dihydroxyphenyl)-1,2,3,4-tetrahydroisoquinoline structure on dopamine sensitive rat retinal adenylate cyclase activity. *Soc. Neurosci. Abstr.* **11**: 315, 1985.
- VAN TOL, H. H. M., BUNZOW, J. R., GUAN, H. C., SUNAHARA, R. K., SEEMAN, P., NIZNIK, H. B. AND CIVELLI, O.: Cloning of the gene for a human dopamine D₂ receptor with high affinity for the antipsychotic clozapine. *Nature* **350**: 610-614, 1991.
- WYRICK, S. D. AND MAILMAN, R. B.: Tritium labelled ($\pm$)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (SCH23390). *J. Labeled Compd. Radiopharm.* **22**: 189-195, 1985.
- ZHOU, Q. Y., GRANDY, D. K., THAMBI, L., KUSHNER, J. A., VAN TOL, H. H. M., CONE, R., PRIBNOW, D., SALON, J., BUNZOW, J. R. AND CIVELLI, O.: Cloning and expression of human and rat D₁ dopamine receptors. *Nature* **347**: 76-80, 1990.

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