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Binding of a Conformationally Restricted Dopamine Analogue, 2-Amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene, to Receptors on Rat Brain Synaptic Membranes

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SUMMARY

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Specific binding of the labeled dopamine analogue 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene to highly purified rat striatal synaptic membranes appears to be closely associated with binding sites for dopamine. The specific binding of the analogue is saturable, with an apparent dissociation constant of 1.2 μM, which correlates with the concentration required to produce half-maximal stimulation of the striatal dopamine-sensitive adenylate cyclase. Binding was enriched in the synaptic membrane fraction, and the regional distribution of binding correlated with the known distribution of dopamine and its receptors. Displacement of binding occurred in the presence of dopamine and the potent neuroleptic *cis*-flupenthixol. The possible nature of the receptor for 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene on synaptic membranes is discussed.

INTRODUCTION

One of the most fruitful approaches to receptor topography has involved the use of conformationally restricted analogues of the hormone or neurotransmitter under investigation. From evidence of structure-activity relationships at dopamine receptors on identified neurons of the snail, *Helix aspersa*, it has been proposed (1) that the Tetralin derivative 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene, which contains the dopamine skeleton held in a rigid cyclic conformation (Fig. 1), should be a potent agonist. Recently it has been demonstrated that ADTN[1] is indeed effective at both invertebrate (2) and vertebrate (3, 4) dopamine receptors: in the rat it causes a long-lasting stimulation of locomotor activity following intraventricular injection (5, 6), and it is equipotent with dopamine in its ability to activate striatal dopamine-sensitive adenylate cyclase (7, 8).

Recently, successful attempts have been made to assay directly receptor sites for a variety of neurotransmitters and pharmacologically active agents (9), using suitable radiolabeled ligands and a variety of membrane preparations. We have therefore obtained [^{3}H]ADTN and have investigated its ability to bind to highly purified synaptic membranes prepared from rat striatum, with the aim of obtaining additional evidence for the involvement of dopamine receptors in the pharmacological actions of ADTN.

During the progress of this work, two

[1] The abbreviation used is: ADTN, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene.

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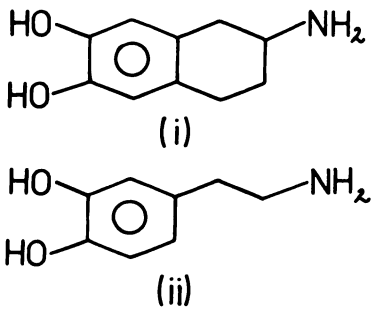

FIG. 1. *Structural formulae of 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (i) and dopamine (ii)*

reports have appeared describing the binding of labeled dopamine to striatal membranes derived from crude synaptosomes (10) or from whole tissue homogenates (11). These authors found that specific [^{3}H]dopamine binding was apparently associated with postsynaptic receptor sites, since neuroleptics were effective inhibitors of binding. However, a possible source of difficulty in the interpretation of data derived from the use of the natural ligand combined with crude biological preparations is the presence of additional specific binding sites, such as enzymes or transport systems, for which the ligand may have equal, if not greater, affinity than for the genuine receptor; thus the development of additional ligands to study dopamine receptors is desirable.

MATERIALS AND METHODS

Preparation of purified synaptic membranes. Striata from rat brain were used, since this region contains the greatest abundance of dopamine, associated with the nerve terminals. Tissues from 30–40 male Wistar rats (450 g) were homogenized in 10 volumes of 0.30 M sucrose in a loosely fitting Teflon-Perspex homogenizer (800 rpm). The homogenate was centrifuged at 1000 × *g* for 20 min to remove nuclei and unbroken cells, and the supernatant was then recentrifuged at 17,000 × *g* for 20 min. The resulting pellet was washed in a small volume of 0.30 M sucrose and resuspended in hypotonic buffer (5 mM Tris, pH 8.1). Rehomogenization in a closely fitting Teflon-glass hand homogenizer was followed by rapid freezing and thawing and incubation at 0° for 30 min to lyse the crude (P_2) synaptosome preparation. The lysate was transferred to a centrifuge tube, and 1.83 M sucrose was added to yield a sucrose concentration of approximately 1.0 M. Bands of 0.83 M and 0.30 M sucrose were layered successively on top of the 1.0 M band, and the gradients were centrifuged in a Beckman SW 27 swing-out rotor for 110 min at 60,000 × *g*. The purified synaptic membranes were harvested from the 0.83/1.0 M interface and resuspended in Tris-Krebs buffer, pH 7.4 (NaCl, 114 mM; KCl, 4.82 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 2.58 mM; $MgSO_4$, 1.2 mM; Tris, 48.5 mM), at a final protein concentration of 4–7 mg/ml. The synaptic membranes were stored at −40° until used; there was no appreciable loss of binding activity for at least 6 weeks of storage under these conditions.

ADTN binding assay. [*G*-^{3}H]ADTN was prepared by the Radiochemical Centre, Amersham, by the process of catalytic exchange labeling, using 100 Ci of $[^3H]_2O$, and was supplied as a crude aqueous solution containing 800 μCi/ml. The label was stored at −140° until required. Immediately before use, the [^{3}H]ADTN was purified by one-dimensional thin-layer chromatography on silica gel plates with 1-butanol–water–acetic acid (12:5:3) as solvent system. Two main bands were obtained, and the one corresponding to authentic, co-chromatographed unlabeled ADTN was scraped off and eluted with ethanol. Following evaporation of solvent, the labeled ADTN was redissolved in distilled water containing 5 mM tartrate, and the concentration of ADTN was determined on the rat anococcygeus muscle preparation (12), which contracts in response to ADTN and other dopamine agonists. The approximate specific radioactivity of the [^{3}H]ADTN was found to be 105 mCi/mmole.

The [^{3}H]ADTN binding assays were performed using a rapid centrifugation method. Incubations of the synaptic membranes were carried out in the absence and presence of a large excess (1 mM) of unlabeled ADTN. The specific [^{3}H]ADTN binding was obtained by subtraction. The incu-

bation mixture consisted of 20 μl of [³H]ADTN (≅ 4 μM) and 70 μl of Tris-Krebs buffer (pH 7.4) containing 3 mM tartrate; drugs, when added, were dissolved in the Tris-Krebs buffer. After a 2-min incubation period, the reaction was started by the addition of 10 μl of synaptic membranes (40–70 μg of protein). After 5 min of incubation at 27°, the tubes were rapidly centrifuged in a Beckman Microfuge, the supernatants were aspirated, and the pellets were solubilized in 400 μl of Soluene-350 (Packard) and assayed for tritium by liquid scintillation counting in 5-(4-biphenylyl)-2-(4-*tert*-butylphenyl)-1-oxa-3,4-diazole in toluene as scintillant. Radioactivity was corrected for quenching, background, and machine efficiency in all cases.

RESULTS

Saturability of binding sites. The specific binding of [³H]ADTN to synaptic membranes represented 10–20% of the total radioactivity bound. It was found to be saturable (thus indicating a finite number of receptor sites), with half-maximal binding at about 1 μM. This contrasted with the effect of increasing [³H]ADTN concentrations on the nonspecific binding component, which increased linearly over the entire concentration range studied (0.5–10 μM) (Fig. 2). Specific binding was found to be a linear function of protein concentration within the range 20–100 μg.

Time course of [³H]ADTN binding. At 27°, specific [³H]ADTN binding approached maximum after approximately 5 min; at 0°, it was very much slower (not shown). Nonspecific binding, however, was not time-dependent and appeared to be essentially instantaneous (Fig. 3).

Kinetics of binding and relationship to adenylate cyclase. Lineweaver-Burk analysis of the specific binding of [³H]ADTN revealed a linear plot (Fig. 4) yielding an apparent dissociation constant (K_D) of 1.20 ± 0.29 μM; i.e., binding within this concentration range apparently occurred to a single population of receptors with medium affinity for the ligand. This value for the dissociation constant is close to that for half-maximal stimulation (3 μM) of the striatal adenylate cyclase system by both ADTN and dopamine (Fig. 5).

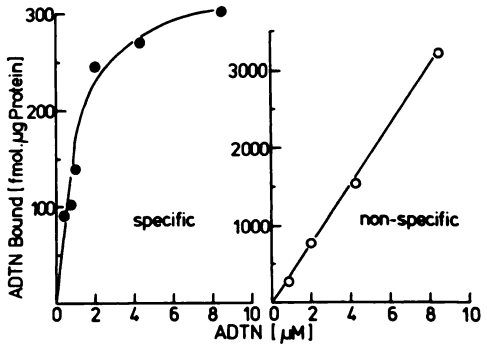

FIG. 2. *Saturability of specific [³H]ADTN binding*

Increasing concentrations of [³H]ADTN were incubated with striatal synaptic membranes, equivalent to 40 μg of protein, in the absence and presence of 1 mM unlabeled ADTN. The results obtained in the presence of the unlabeled ADTN represent nonspecific binding. Saturation of specific binding occurs over a fairly narrow concentration range (0.5–10 μM). Each result is the mean of at least four replicate assays, and the difference between individual observations was less than 15%.

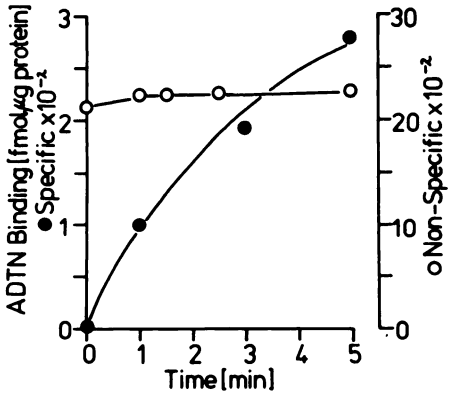

FIG. 3. *Time course of [³H]ADTN binding*

The rate of binding at 27° of [³H]ADTN to striatal synaptic membranes was determined as described in the text. Each point is the mean of triplicate determinations.

Subcellular distribution of binding sites. To determine the relative ability of various subcellular fractions to bind [³H]ADTN specifically, striata were homogenized in 0.32 M sucrose at 0° and subjected to differential and density gradient centrifugation according to Gray and Whittaker (15). Each fraction was washed with an excess of Tris-Krebs buffer to re-

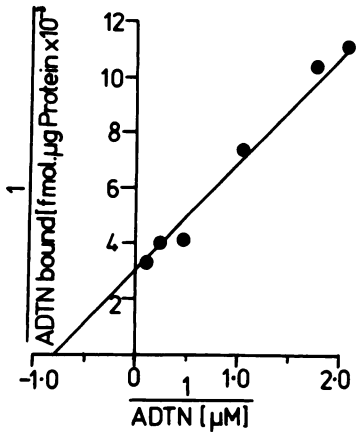

FIG. 4. *Lineweaver-Burk plot of specific [³H]ADTN binding to striatal synaptic membranes in Tris-Krebs buffer*

The line of best fit was determined by regression analysis. Each point is the mean of quadruplicate determinations. ADTN bound is expressed as femtomoles per microgram of protein.

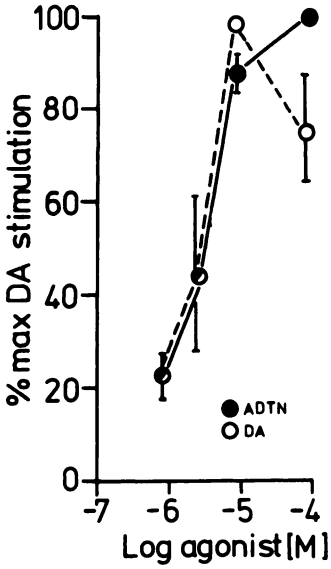

FIG. 5. *Activity of ADTN and dopamine on rat striatal adenylate cylase*

The potency of each agonist in increasing cyclic AMP production in rat striatal homogenates was determined by the method of Kebabian *et al.* (13), and the resulting cyclic AMP was measured by a protein binding assay (14). Potency is expressed as a percentage of the maximum responses produced by 100 μM dopamine. From the log dose-response curve the EC_{50} value is 3.2 ± 1.0 μM, with a similar value for ADTN. Each point is the mean ± standard error from four to eight estimations.

move any interfering endogenous amines. ADTN binding was examined in six fractions: crude homogenate, crude nuclear pellet, P_2 fraction, myelin fraction, crude synaptosome fraction, and mitochondrial fraction (Table 1). Total binding was greatest in the P_2 fraction and least in the whole homogenate. However, specific binding was detected in only three fractions—a small component in the myelin fraction, but principally in the P_2 and lysed synaptosome fractions, the latter binding approximately 3 times the amount of [³H]ADTN bound to the P_2 fraction.

Regional distribution of specific [³H]ADTN binding in central nervous system. In general, neurotransmitters exhibit a heterogeneous distribution throughout the central nervous system, and this is reflected by regional variations in the density of receptor sites. Frequently, though not invariably, a high receptor density is accompanied by high concentrations of the relevant neurotransmitter. In the case of dopamine, its endogenous levels, and the dopamine-sensitive adenylate cyclase are localized particularly within the corpus striatum of the extrapyramidal system and in the so-called limbic region. Some preliminary evidence (16) suggests that dopamine binds preferentially to these areas, although the extent of binding was very low. Specific [³H]ADTN binding was detected in each of the five regions of rat

TABLE 1

Subcellular distribution of [³H]ADTN binding sites

Subcellular fractions were prepared from rat striata as described in the text, and [³H]ADTN binding was determined. Data represent the means of three experiments, whose results varied less than 20%.

Fraction	Total binding	Nonspecific binding	Specific binding
	fmoles/µg protein		
Whole homogenate	368.43	373.40	
Crude nuclear pellet	424.97	455.77	
P_2	594.51	562.08	32.43
Myelin	564.31	551.54	12.77
Lysed synaptosomes	473.79	372.72	101.07
Mitochondrial	455.56	455.34	

brain investigated, and there was an indication of some regional differences, in that specific binding was greatest in synaptic membranes derived from striatum and substantially less in the other brain areas (Table 2). Extensive binding of ADTN occurred with plasma membranes isolated from rat kidney, an organ rich in dopamine receptors associated with the vasculature and where ADTN has potent renal and mesenteric vasodilator actions (17).

Effects of drugs on specific [^{3}H]ADTN binding. If indeed specific [^{3}H]ADTN binding sites are associated with striatal dopamine receptors, binding of the ligand should be inhibited both by dopamine and its congeners and by the neuroleptic class of drugs, which are thought to produce their therapeutic effects by dopamine receptor blockade. In this respect, it is particularly interesting that different stereoisomers of the various neuroleptics differ very widely in their therapeutic efficacy. For example, the thioxanthene *cis*-flupenthixol is a potent antischizophrenic agent, while the *trans* isomer is clinically ineffective.

In a preliminary study, the binding of [^{3}H]ADTN (4 μM) in the presence of a large molar excess of the drug under investigation was measured (Table 3). Specific binding was totally abolished in the presence of 1 mM ADTN or dopamine. A 10-fold increase in the concentration of ADTN produced no reduction in the nonspecific binding component, indicating that the method employed for determining specific [^{3}H]ADTN binding was valid. There were marked differences in the ability of the two isomers of flupenthixol (1 mM) to affect the binding; the *trans* isomer was completely ineffective, while the pharmacologically active *cis* form was approximately half as effective at this concentration as was ADTN itself in displacing [^{3}H]ADTN binding. The potent dopamine uptake blocker benztropine, which has negligible affinity for the receptors, produced a very modest inhibition of specific binding, while two other putative neurotransmitters, histamine and glycine, were inactive. In further experiments, the specific binding of [^{3}H]ADTN was studied in the presence of a range of concentrations of dopamine, ADTN, *cis*-flupenthixol, and the dopamine agonist epinine (methyldopamine). Plots were drawn of inhibitor concentration (logarithmic scale) against [^{3}H]ADTN specific binding, as a percentage of control on a probability scale, in order to obtain the IC_{50} value (inhibitor concentration producing 50% inhibition of binding) (Table 4). All the plots were parallel (not shown) and displaced toward higher inhibitor concentrations as potency decreased, thereby suggesting that each of the substances tested was a competitive inhibitor of specific ADTN binding. Although ADTN and epinine are roughly equipotent with dopa-

TABLE 2

Regional distribution of specific [^{3}H]ADTN binding to synaptic membranes

Specific binding of [^{3}H]ADTN (4 μM) was determined as described in the text. Results are means of three observations ± standard errors.

Region	Specific binding
	fmoles/μg protein
Striatum	247 ± 74
Cerebral cortex	60 ± 24
Cerebellum	87 ± 13
Pons-medulla	139 ± 2
Spinal cord	103 ± 12
Kidney	305 ± 28

TABLE 3

Drug competition for specific [^{3}H]ADTN binding

The binding of [^{3}H]ADTN (4 μM) to striatal synaptic membranes (equivalent to 40 μg of protein) was determined in the presence of an excess of the drug under investigation. Values are means ± standard errors for the number of experiments shown in parentheses.

Compound	Specific binding		Inhibition
	fmoles/μg protein		%
None	239 ± 28	(10)	
ADTN (1 mM)	26 ± 3	(6)	89
ADTN (10 mM)		(5)	100
Dopamine (1 mM)		(4)	100
cis-Flupenthixol (1 mM)	115 ± 33	(4)	52
trans-Flupenthixol (1 mM)	247 ± 30	(4)	
Benztropine	188 ± 66	(6)	21
Glycine (1 mM)	233 ± 69	(4)	3
Histamine (1 mM)	217 ± 217	(4)	9

mine in their ability to activate striatal dopamine-sensitive adenylate cyclase (2), in terms of their ability to inhibit specific ADTN binding to synaptic membranes they both had approximately one-third the potency of dopamine. The neuroleptic *cis*-flupenthixol was considerably less effective than the three agonists in antagonizing specific binding.

Chemical nature of ADTN receptor. The thermal stability of specific [^{3}H]ADTN binding was examined by incubating the synaptic membranes for 10 min at 70° before the standard binding assay at 27°. This treatment was found to abolish completely the specific binding and to reduce significantly the nonspecific component (Table 5). Prior treatment with a low concentration (0.04%) of Triton X-100 was found to enhance the ADTN binding capacity approximately 3.6-fold; a similar finding has been reported for the binding of [^{3}H]strychnine to membranes prepared from rat spinal cord (18). Sodium deoxycholate (0.04%) had a minimal effect on specific binding. The binding component was also found to be sensitive to proteolytic enzymes and to exposure to concanavallin A (0.1 mg/ml). The effects of these agents suggest that the ADTN receptor may be a membrane protein or glycoprotein, although involvement of phospholipids is also possible.

TABLE 4

Displacement of specific [^{3}H]ADTN binding from rat striatal synaptic membranes

Synaptic membranes (equivalent to approximately 40 μg of protein) were first incubated for 10 min with the drug under investigation at a concentration of 10–1000 μM. [^{3}H]ADTN (4 μM) was added to start the reaction, and the binding assay was performed as described in the text. Log dose inhibitor concentration was plotted against specific binding as a percentage of control, and the IC_{50} values were read. Each point on the plots (not shown) represented the mean of three determinations.

Drug	IC_{50}
	μM
ADTN	30
Dopamine	9.3
cis-Flupenthixol	178
Epinine	45

TABLE 5

Effects of various chemical treatments on specific [^{3}H]ADTN binding

Synaptic membranes were first incubated for 10 min at 27° in the presence of the relevant agent, and the binding assay was started by the addition of [^{3}H]ADTN (4 μM) and continued for a further 5 min. The results are the means of triplicate determinations (standard errors were less than 20%).

Treatment	Binding (average)
	% control
Heat treatment (70°)	
Sodium deoxycholate (0.04%)	86
Triton X-100 (0.04%)	353
Trypsin (0.4 mg/ml)	23
Concanavalin A (0.1 mg/ml)	53

DISCUSSION

The specific binding of [^{3}H]ADTN to highly purified rat striatal synaptic membranes reported in this study appears to be associated with binding sites for dopamine, since ADTN was readily displaced by dopamine itself and also by the potent neuroleptic and dopamine receptor antagonist *cis*-flupenthixol.

Although the effects of the various drugs tested on specific [^{3}H]ADTN binding were at least qualitatively similar to their effects on dopamine binding to crude synaptic membranes (10, 11), the essential difference found was in the affinities of the ligands for their receptors. The apparent dissociation constants for dopamine (10, 16) were in the range 10–25 nM, while that for ADTN was approximately 100 times greater (1.2 μM). However, the dopamine binding studies were carried out with ligand concentrations restricted to the nanomolar range, and any additional saturable binding component of lower affinity, such as that seen for [^{3}H]ADTN, would not have been detected; it is interesting that in the case of [^{3}H]haloperidol binding there appeared to be multiple binding sites. Since neuroleptics appear to exhibit a much greater affinity for the dopamine-sensitive adenylate cyclase (50–100-fold) than for the dopamine binding sites, it has been proposed that the receptor coexists in "agonist" and "antagonist" configurations (16). However, Seeman *et al.* (10) have

detected no difference between the dopamine and haloperidol binding sites. Another major discrepancy in the binding of [^{3}H]dopamine has been the poor correlation between the affinity of dopamine for its receptor and for the adenylate cyclase: on the cyclase, its affinity is 100 times less than that for the binding site (16). In this respect, it is pertinent that our study revealed a K_D for ADTN very close to the EC_{50} (3 μM) for both ADTN and dopamine on the enzyme.

It seems likely that the binding of [^{3}H]ADTN may represent an interaction with dopamine receptors, since binding not only was highly localized to synaptic membrane fractions but exhibited a regional distribution compatible with this hypothesis. However, it is necessary to interpret data derived from all binding studies using a heterogeneous preparation with caution; it has been demonstrated in the past (19) that binding occurs to other membrane components. Although ADTN is not a substrate for monoamine oxidase, it would be expected to bind to catechol *O*-methyltransferase, which can exist in a membrane-bound form (20), and possibly also to combine with synaptosomal transport sites for dopamine (21). Current experiments are aimed at elucidating these possibilities.

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