STRIATAL BINDING OF 2-AMINO-6,7-³H-DIHYDROXY-1,2,3,4-TETRAHYDRONAPHTHALENE TO TWO DOPAMINERGIC SITES DISTINGUISHED BY THEIR LOW AND HIGH AFFINITY FOR NEUROLEPTICS¹

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Abstract

In order to develop more selective methods for labeling brain dopamine receptors, this study describes in detail the properties of 2-amino-6,7-³H-dihydroxy-1,2,3,4-tetrahydronaphthalene (³H)ADTN) binding to dopaminergic sites in rat, calf, and human brain.

³H)ADTN labeled two distinct types of dopaminergic binding sites in the brain striatum of the rat, calf, and human. Very low concentrations of dopamine and dopaminergic catecholamines (with IC₅₀ values of 1 to 10 nM) inhibited the binding of ³H)ADTN to both sites. Neuroleptics, however, inhibited the binding of ³H)ADTN in two distinctly separate concentration ranges, with IC₅₀ values of 0.15 to 40 nM at one site and 100 to 50,000 nM at the other site. The site with high affinity for dopamine and low affinity for neuroleptics had binding properties that corresponded to those of the previously characterized D₃ site (List, S., M. Titeler, and P. Seeman (1980) *Biochem. Pharmacol.* 29: 1621-1622). The ³H)ADTN binding site with high affinity for neuroleptics demonstrated binding characteristics similar to a site labeled by ³H-neuroleptics (Sokoloff, P., M. P. Martres, and J. C. Schwartz (1980) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 315: 89-102). ³H)Apomorphine appeared to label the same two sites as ³H)ADTN, while ³H)dopamine labeled only the D₃ site. Scatchard analysis of ³H)ADTN or ³H)apomorphine binding, under conditions for selective labeling of the low affinity neuroleptic site (D₃) and the high affinity site for neuroleptics, detected a density of 70 fmol/mg of protein for each. The density of the D₃ site in the calf striatum (170 fmol/mg of protein) was much greater than that of the high affinity neuroleptic site (50 fmol/mg). In the rat, the dissociation constant (*K_D*) of ³H)ADTN was 2 nM for both sites. ³H)Apomorphine, however, had a higher affinity for the D₃ site (*K_D* = 1.6 nM) than for the high affinity neuroleptic site (*K_D* = 4.2 nM). The present results may explain previously observed species and laboratory differences between the binding of ³H)ADTN, ³H)apomorphine, and ³H)dopamine.

In order to develop more selective methods for labeling brain dopamine receptors, this study describes in detail additional properties of 2-amino-6,7-³H-dihydroxy-1,2,3,4-tetrahydronaphthalene (³H)ADTN) binding to dopaminergic sites in rat, calf, and human brain.

D₁ dopaminergic sites have been defined by their ability to stimulate adenylate cyclase (Kebabian and Calne, 1979), and these sites have been labeled directly by *cis*-³H)flupenthixol and ³H)piflutixol (Hyttel, 1978, 1981; Cross and Owen, 1980).

D₂ dopamine receptors were defined as those which do

not stimulate adenylate cyclase (Kebabian and Calne, 1979) but which now appear to be linked to the inhibition of adenylate cyclase (Munemura et al., 1980; Cote et al., 1981; Meunier and Labrie, 1981). D₂ receptors have been labeled by ³H-neuroleptics (of the butyrophenone class) and ³H-ergots (Burt et al., 1976; Seeman et al., 1976b; Leysen et al., 1978; Caron et al., 1978; Baudry et al., 1979). D₂ receptor sites generally exhibited nanomolar (i.e., high affinity) dissociation constants for these ³H-ligands but were occupied only by micromolar (i.e., low affinity) concentrations of the agonists dopamine, apomorphine, and ADTN ((±)-6,7-dihydroxy-2-amino-Tetralin). Studies have indicated, however, that ³H-butyrophenone neuroleptics also label sites with *high affinity* for *both* neuroleptics and dopamine agonists. The two sites have been called D₂ and D₄ (List and Seeman, 1981), respectively (or D-4 and D-2 (Sokoloff et al., 1980b) or

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D₂ low and D₂ high (Sibley et al., 1981)). It has been suggested that these two apparent binding sites (labeled by ³H-butyrophenones) actually may be different states of the same D₂ receptor (Sibley et al., 1981).

A third distinct dopaminergic binding site, which we now call D₃ (Titeler et al., 1979; List et al., 1980), has been detected in the calf caudate using [³H]dopamine, [³H]apomorphine (Seeman et al., 1975, 1976b; Burt et al., 1975, 1976), and [³H]ADTN (Seeman et al., 1979; Cross et al., 1979). This site is characterized by its high affinity (with *K_D* values of 1 to 10 nM) for dopamine, as well as other dopaminergic catecholamines, such as apomorphine and ADTN, and its low affinity for potent neuroleptics (with *K_D* values of 100 nM and greater). We have reported recently that a binding site with D₃ properties can be detected in dopaminergic areas of the rat and human brain, as well as the calf brain, using [³H]dopamine (List et al., 1980).

[³H]Apomorphine and [³H]ADTN, however, often have appeared to label sites in the rat brain different from those detected in the calf (Creese and Snyder, 1978; Creese et al., 1978, 1979). One of the problems with [³H]apomorphine and [³H]ADTN is that these ligands appear to label multiple sets of binding sites in rat brain. Using [³H]ADTN and [³H]apomorphine, Creese et al. (1978, 1979), for example, have demonstrated biphasic drug competition curves against these ligands. In these studies, however, no attempt was made to label selectively and characterize the binding of these ligands to separate populations of sites. Drug IC₅₀ values reported by these workers thus represent a combination of the IC₅₀ values of different populations of [³H]apomorphine or [³H]ADTN binding sites. Recently, Sokoloff et al. (1980a, b) have demonstrated that [³H]apomorphine in rat striatum can bind to the D₃ site as well as to another distinctly different site characterized by a high affinity for both dopamine and dopaminergic catecholamines and neuroleptics.

We have now re-examined [³H]ADTN binding in order to label selectively and characterize the various dopaminergic binding sites labeled by this ligand in rat, calf, and human brain.

Materials and Methods

Tissue preparation. Brains from calves (Hunnisett plant, Canada Packers Co.) or from rats (male Wistar, 200 to 250 gm, sacrificed by cervical dislocation) were removed immediately after death, stored in ice (calf) or in ice cold saline (rat), and dissected within 1 to 2 hr after death. Neurologically normal human brains were obtained from individuals (22 to 91 years of age) who had died from pneumonia or myocardial infarction or in car accidents. After removal from the cranium (3 to 25 hr postmortem), human brains were stored frozen. For dissection, human brains were removed from storage and allowed to reach approximately 0°C to allow the cutting of coronal sections. The sections then were dissected on glass plates over dry ice.

Dissected regions from brains of calf, rat, and human were placed in 15 vol of cold TEAN buffer (15 mM Tris-HCl, pH 7.4, 5 mM Na₂EDTA, 0.02% (1.1 mM) ascorbate, and 12.5 μM nialamide), briefly Teflon glass homogenized,

centrifuged (44,000 × *g*), resuspended (15 vol) four times (in order to remove endogenous dopamine), and finally stored frozen (−20°C, 3- to 5-ml aliquots of 180 to 300 (calf), 35 (rat), or 40 mg (human), original wet weight/ml). Before final use, the suspension was diluted (calf only) and Polytron homogenized (Brinkmann PT-10, setting of 7, 10 sec).

Binding studies. Assays were carried out using quadruplicate glass test tubes (12 × 75 mm) which received in order: 200 μl of buffer (with or without competing non-radioactive drug), 200 μl either of [³H]ADTN (25 to 35 Ci/mmol, New England Nuclear, Boston) or [³H]apomorphine (39 Ci/mmol, New England Nuclear, Boston), and 200 μl of tissue (0.3 to 0.4 mg of protein for calf; 0.4 to 0.5 mg of protein for rat, or 0.5 mg of protein for human). After incubation at 20 to 22°C for 30 min, 0.5-ml aliquots were vacuum-filtered through Whatman GF/B filters, followed by a 10-ml wash with buffer. Filters were inserted into liquid scintillation vials along with either 8 ml of Aquasol (New England Nuclear, Boston) or ACS (Amersham, Arlington Heights, IL) and equilibrated for at least 12 hr before the determination of radioactivity.

Unless specified, all results expressed were averaged for two to five individual experiments. Binding isotherms and Scatchard analyses were done using [³H]ADTN, [³H]dopamine, and [³H]apomorphine in concentrations of 0.2 to 6.0 nM. The specific binding of [³H]ADTN, [³H]apomorphine, and [³H]dopamine was defined as that binding displaceable by 1 μM dopamine (for [³H]apomorphine assays) or 1 μM apomorphine (for [³H]ADTN and [³H]dopamine assays). Selective ³H-ligand binding to the population of sites with high affinity for both dopaminergic catecholamines and neuroleptics was defined as that binding displaceable by 30 nM spiperone (see Fig. 3 under "Results"). Selective binding to the population of sites with high affinity for dopaminergic catecholamines and low affinity for neuroleptics (D₃ sites) was defined as that binding occurring in the presence of 30 nM spiperone (to occlude the high affinity sites for neuroleptics) and displaceable by 1 μM apomorphine or dopamine. In drug competition experiments against ³H-ligand binding, drug IC₅₀ values were defined as that concentration of drug required to inhibit 50% of the specific binding. If drugs exhibited two clearly separate high affinity and low affinity phases in their competition for specific ³H-ligand binding, separate IC₅₀ values were determined for each phase of competition. IC₅₀ values for the first and second phases of competition were defined as those drug concentrations required to inhibit 50% of the high affinity phase binding and 50% of the low affinity phase binding, respectively. Hill coefficients for drug competition curves were determined from the slope (*m*) of the line:

$$\log [\text{drug}] = m \times \log \frac{\% \text{ inhibition of specific binding}}{100 - \% \text{ inhibition of specific binding}}$$

Results

Drug competition in rat striatum. A variety of drugs were tested for their ability to compete with the binding of 0.9 nM [³H]ADTN to rat striatum. As shown in Figures 1, 2 (top), and 3 (top), a number of drugs competed

Figure 1. Competition of dopaminergic catecholamines, ergots, neuroleptics, and adrenergic compounds against the binding of $0.9 \text{ nM } [^3\text{H}]\text{ADTN}$ to homogenates of rat striatum. Binding to the filter comprised 40 cpm. The points are averages ($\pm \text{SEM}$) for three to five experiments.

Figure 2. *Top*, Competition of dopaminergic catecholamines, ergots, and neuroleptics against the binding of $0.9 \text{ nM } [^3\text{H}]\text{ADTN}$ to homogenates of rat striatum. Many neuroleptics produced pronounced two-phase competition curves, while dopaminergic catecholamine agonists and ergots competed for radioligand binding in one continuous phase. See Figure 1 for additional details. *Bottom*, Competition for dopaminergic catecholamines, ergots, and neuroleptics against the binding of $0.9 \text{ nM } [^3\text{H}]\text{ADTN}$, in the presence of 30 nM spiperone, to rat striatal homogenates. Compared to the *top panel*, dopaminergic catecholamine curves are steeper and neuroleptic curves are no longer biphasic. The points are averages ($\pm \text{SEM}$) for two to five experiments.

Figure 3. Competition of neuroleptics against the binding of 0.9 nM [^3H]ADTN to homogenates of rat striatum. See Figure 2 for additional details.

effectively for [^3H]ADTN binding, showing competition plateaus at 45 to 50% of the total binding. ADTN, itself, however, displaced more [^3H]ADTN than the other drugs, as usually found in self-competition data (Seeman, 1980). As shown in Table I, column 2, the dopamine catecholamine agonists (NPA (*N*-propyl norapomorphine), ADTN, apomorphine, and dopamine) were most potent at inhibiting specific binding of [^3H]ADTN (with IC_{50} values of 3 to 10 nM), while the dopaminergic ergots (LSD (lysergic acid diethylamide), DHEC (dihydroergocryptine), and bromocryptine) were somewhat less potent (with IC_{50} values of 13 to 60 nM). Neuroleptic drugs showed a wide range of IC_{50} values; piflutixol had the lowest IC_{50} value (13 nM), while drugs such as metoclopramide and domperidone had IC_{50} values of greater than 1000 nM. While norepinephrine was quite potent at inhibiting [^3H]ADTN binding, both α - and β -adrenergic antagonists were very weak at inhibiting binding.

Competition curves against [^3H]ADTN, particularly those of neuroleptics, had Hill coefficients significantly less than unity (Table I). Such Hill coefficients can indicate the presence of either negative cooperativity or multiple sites for ligand binding. However, it is strongly suggested from the biphasic competition curves of domperidone, spiperone, sulpiride, and metoclopramide (Figs. 1 and 3), which showed a clear separation between the high affinity and low affinity phases of competition, that [^3H]ADTN was labeling more than one binding site. These neuroleptics and others (not shown) demonstrated high affinity competition leveling at 72 to 78% of the total binding, followed by a lower affinity competition phase leveling at 45 to 50% of the total binding. Thus, 0.9 nM [^3H]ADTN appeared to label at least two sites; almost half of the specific binding was associated with a high affinity site for neuroleptics, while the remaining specific binding was associated with a low affinity site for neuroleptics. Table II lists IC_{50} values at the two sites for neuroleptics having clearly separate high affinity and low affinity phases of competition.

As shown in Figures 1, 2 (top), and 3 (top), the catecholamines, ergot dopamine agonists, and some neuro-

TABLE I
 IC_{50} values against [^3H]ADTN (0.9 nM) binding to rat striatum

Drug	In Absence of 30 nM Spiperone		In Presence of 30 nM Spiperone	
	IC_{50}^a	Hill Coefficient	IC_{50}^a	Hill Coefficient
	nM		nM	
NPA	3.0 ± 1.0	0.78		
($\pm$)-ADTN	3.9 ± 0.4	0.66	4.5 ± 2.0	0.92
Apomorphine	4.0 ± 0.9	0.77	2.2 ± 0.6	0.92
Dopamine	10 ± 2.0	0.68	5.0 ± 1.6	1.00
Norepinephrine	35 ± 10.0	0.68		
<i>d</i> -LSD	13 ± 0.9	0.78		
DHEC	35 ± 3.0	0.79		
Bromocryptine	58 ± 7.0	0.62	317 ± 45	0.97
Piflutixol	13 ± 1.0	0.63		
Fluphenazine	43 ± 10.0	0.51		
<i>cis</i> -Flupenthixol	55 ± 5.0	0.77	417 ± 74	0.77
(+)-Butaclamol	63 ± 8.0	0.61	90 ± 10	0.62
Haloperidol	108 ± 34.0^b	0.41	412 ± 116	1.00
Chlorpromazine	333 ± 77.0^b	0.51	767 ± 150	0.6
Spiperone	425 ± 116^b	0.23	$5,000 \pm 1,000$	0.8
($\pm$)-Sulpiride	600 ± 350^b	0.29	$9,000 \pm 1,000$	
Metoclopramide	$>10,000^b$	0.29	$>10,000$	
Domperidone	$>40,000^b$	0.20	$>40,000$	
Phentolamine	$2,500 \pm 700$	0.84		
Isoproterenol	$>10,000$			

^a IC_{50} value was defined as that drug concentration required to inhibit 50% of all specific binding. Specific binding of [^3H]ADTN in the presence or absence of 30 nM spiperone was defined as that binding displaceable by 1 μM apomorphine (see Fig. 2). The values are the mean $\pm$ SEM.

^b Biphasic competition curves.

leptics (e.g., fluphenazine, *cis*-flupenthixol, and (+)-butaclamol), unlike most neuroleptics, showed shallow competition curves rather than two clearly separate phases of competition. Such behavior may indicate that these

compounds competed for more than one $[^3\text{H}]\text{ADTN}$ binding site but did not have sufficiently different affinity for these sites to produce two clearly separate phases of competition. The relatively low IC_{50} values (3 to 10 nM) and the Hill coefficients of approximately 0.7 to 0.8 for the dopaminergic catecholamine agonists suggest that these compounds had a high affinity for all specific $[^3\text{H}]\text{ADTN}$ binding sites.

Although dopamine competed relatively effectively for the binding of $[^3\text{H}]\text{apomorphine}$ (Fig. 4, *top*), the competition curve was shallow (Hill coefficient of 0.71). Neuroleptic competition against $[^3\text{H}]\text{apomorphine}$ showed

TABLE II
Binding of $[^3\text{H}]\text{ADTN}$ to rat striatum: High affinity and low affinity sites for neuroleptics

Drug	IC_{50}^a	
	High Affinity Site	Low Affinity Site
	nM	
Spiperone	0.15	2,800
Domperidone	1.25	>10,000
Sulpiride	5.0	25,000
Haloperidol	15.0	2,000
Metoclopramide	20.0	>50,000
Chlorpromazine	42.0	2,300

^a IC_{50} value was defined as that drug concentration required to inhibit 50% of specific binding. The drugs listed showed two distinctly separate phases of inhibition of $[^3\text{H}]\text{ADTN}$ binding. Specific binding to the high affinity site for neuroleptics was defined by the plateau of the first phase of inhibition occurring at 78 to 72% of the total $[^3\text{H}]\text{ADTN}$ binding. Specific binding to the low affinity site for neuroleptics was defined as the binding between the first plateau of inhibition and the second plateau of inhibition (occurring at 45% to 50% of total $[^3\text{H}]\text{ADTN}$ binding) (see Figs. 1 to 3).

clearly separate high affinity and low affinity phases similar to those described using $[^3\text{H}]\text{ADTN}$.

In contrast to $[^3\text{H}]\text{apomorphine}$ and $[^3\text{H}]\text{ADTN}$, dopamine was potent against $[^3\text{H}]\text{dopamine}$ binding in the rat striatum with a Hill coefficient of close to unity (Fig. 4, *bottom*). Furthermore, spiperone and sulpiride, neuroleptics which had demonstrated both high affinity and low affinity competition against $[^3\text{H}]\text{ADTN}$ and $[^3\text{H}]\text{apomorphine}$ binding, showed only low affinity competition against the binding of $[^3\text{H}]\text{dopamine}$.

In order to label selectively the sites with high affinity for dopamine and low affinity for neuroleptics using $[^3\text{H}]\text{ADTN}$, assays were done in the presence of 30 nM spiperone (see Fig. 3, *top*) to occlude binding to sites with high affinity for neuroleptics. As shown in Figures 2 and 3 and in Table I, the addition of spiperone to the assay system resulted in steepening of competition curves with an increase in neuroleptic IC_{50} values and either a decrease or no change in the IC_{50} values for catecholamine agonists. In the presence of spiperone, neuroleptic IC_{50} values were similar to those of the low affinity phase IC_{50} values against $[^3\text{H}]\text{ADTN}$ binding in the absence of spiperone. Furthermore, in the presence of spiperone, the pattern of drug IC_{50} values against $[^3\text{H}]\text{ADTN}$ binding was identical to the previously characterized D_3 site (List et al., 1980).

$[^3\text{H}]\text{ADTN}$ binding in calf caudate nucleus. Dopaminergic catecholamines competed effectively for 85% of the total binding of $[^3\text{H}]\text{ADTN}$ (0.8 nM; Fig. 5). As with rat striatum, neuroleptics also competed for this same binding, and many neuroleptics competed for the binding with distinct high affinity and low affinity phases. The IC_{50} values for the two phases are reported in Table III. The high affinity phase in the calf caudate comprised

Figure 4. Competition of dopamine and neuroleptics for the binding of 0.75 nM $[^3\text{H}]\text{apomorphine}$ (*top*) and 0.7 nM $[^3\text{H}]\text{dopamine}$ (*bottom*) to homogenates of rat striatum. Neuroleptic competition curves are monophasic against $[^3\text{H}]\text{dopamine}$ binding and biphasic against the binding of $[^3\text{H}]\text{apomorphine}$. Binding to the filter was 55 cpm for $[^3\text{H}]\text{apomorphine}$ and 50 cpm for $[^3\text{H}]\text{dopamine}$. The points are averages ($\pm$ SEM) for three to five experiments.

15% of the total binding, indicating that about 17% of all specific binding was to the high affinity site for neuroleptics, while the remainder of the binding was to sites with low affinity for neuroleptics. As shown in Figure 5 (bottom) and in Table IV, the addition of 30 nM spiperone to the assay system resulted in the elimination of the high affinity phase of neuroleptic competition and in an increase of the IC_{50} value and Hill coefficient for the neuroleptic, piflutixol. In contrast, the IC_{50} values and Hill coefficients for the catecholamine agonists were little affected.

[3H]ADTN competition curves in human caudate. Dopaminergic catecholamines were very potent in competing (with somewhat shallow curves) for approximately 55% of the total [3H]ADTN binding (Fig. 6). Many neuroleptics (including spiperone and haloperidol) showed distinct high affinity and low affinity phases of competition. The high affinity component accounted for 15% of the total [3H]ADTN binding. Thus, using 0.75 nM [3H]ADTN in the human caudate, approximately 25% of the specific [3H]ADTN binding was to the high affinity site for neuroleptics, while the remaining specific binding was to sites with low affinity for neuroleptics.

The IC_{50} values for drugs against all specific [3H]ADTN binding and against [3H]ADTN binding to the low affinity and high affinity neuroleptic sites in human caudate are shown in Table V.

Figure 5. Competition of dopaminergic catecholamines against the binding of 0.8 nM [3H]ADTN to homogenates of calf caudate in the absence (top) or presence (bottom) of 30 nM spiperone. Top, Many neuroleptics produced pronounced two-phase competition curves, while dopaminergic catecholamines competed for binding in one continuous phase. Bottom, With the addition of spiperone, neuroleptic curves became monophasic and shifted to the right. The points are averages ($\pm$ SEM) for two or three experiments.

TABLE III
[3H]ADTN (0.8 nM) binding to calf caudate: High and low affinity sites for neuroleptics

Drug	All Specific Binding		IC_{50}^a	
	IC_{50}^a	Hill Coefficient	High Affinity Site	Low Affinity Site
	nM		nM	
Haloperidol	680	0.37	2.5	2,500
Spiperone	3,033 $\pm$ 1,950	0.20	0.7	>10,000
Sulpiride	>10,000	0.37	25.0	>10,000

^a IC_{50} value was defined as that drug concentration required to inhibit 50% of specific binding. All specific binding was defined as that amount of [3H]ADTN binding inhibited by 1 μ M apomorphine. This or higher concentrations of apomorphine or other drugs produced a plateau in inhibition occurring at 15% of the total [3H]ADTN binding (see Fig. 5). The drugs listed showed two distinctly separate phases in the inhibition of [3H]ADTN binding. Specific binding to the high affinity site for neuroleptics was defined by the plateau of the first phase of inhibition, occurring at 85% of the total [3H]ADTN binding. Specific binding to the low affinity site for neuroleptics was defined as the binding between the first plateau of inhibition and the second plateau of inhibition (occurring at 15% of the total [3H]ADTN binding).

TABLE IV
[3H]ADTN (0.8 nM) binding to calf caudate

Drug	In Absence of 30 nM Spiperone		In Presence of 30 nM Spiperone	
	IC_{50}^a	Hill Coefficient	IC_{50}^a	Hill Coefficient
	nM		nM	
ADTN	1.0 $\pm$ 1.04 ^b	1.04	2.0 $\pm$ 0.7	1.12
Dopamine	1.8 $\pm$ 0.07	0.84	1.4 $\pm$ 0.2	0.96
Apomorphine	4.5 $\pm$ 0.98	0.98	3.2 $\pm$ 0.7	0.84
Piflutixol	19 $\pm$ 1.4	0.63	240 $\pm$ 57	0.96

^a IC_{50} value was defined as that drug concentration required to inhibit 50% of all specific binding. Specific binding in the presence or absence of 30 nM spiperone was defined as that binding displaceable by 1 μ M apomorphine (see Fig. 4).

^b Mean $\pm$ SEM.

Scatchard analysis of [3H]ADTN, [3H]apomorphine, and [3H]dopamine binding. Binding isotherms and Scatchard analyses were performed under conditions (see "Materials and Methods") which allowed quantitation of the total number of specific binding sites, the number of high affinity sites for neuroleptics, and the number of low affinity sites for neuroleptics labeled by [3H]ADTN, [3H]apomorphine, and [3H]dopamine in calf caudate and rat striatum. The term D_3 was used, as previously (List et al., 1980), to describe those sites with high affinity for dopamine and low affinity for neuroleptics. Representative Scatchard analyses in rat, calf, and human for [3H]ADTN binding to all specific sites, the D_3 sites, and the high affinity sites for neuroleptics are shown in Figure 7. Table VI lists average B_{max} and K_D values for the binding of [3H]dopamine, [3H]apomorphine, and [3H]ADTN to their specific binding sites in rat striatum and calf and human caudate.

In the rat, [3H]ADTN had a similar high affinity for the D_3 site and the high affinity neuroleptic site, while [3H]apomorphine had a somewhat higher affinity for the D_3 site. Regardless of which of these ligands was used, the density for each of both sites in rat striatum was approximately 70 fmol/mg of protein.

Figure 6. Competition of dopaminergic catecholamines and neuroleptics for the binding of 0.75 nM $[^3\text{H}]\text{ADTN}$ to human caudate. Spiperone and haloperidol both produced two distinctly separate phases of competition. Homogenates of caudate nucleus were prepared from the brain of a 45-year-old male 4 hr postmortem. The points are averages ($\pm$ SEM) for two or three experiments.

TABLE V
 IC_{50} values against $[^3\text{H}]\text{ADTN}$ (0.75 nM) binding to human caudate

Drug	All Specific Binding		IC_{50}^a	
	IC_{50}^a	Hill Coefficient	High Affinity Site	Low Affinity Site
	nM		nM	
ADTN	3.5 ± 0.7^b	0.66		
Apomorphine	4.5 ± 1.0	0.88		
Dopamine	12.5 ± 2.5	0.71		
Piflutixol ^c	55 ± 10	0.50	2.0	70
(+)-Butaclamol	100 ± 10	0.51		
cis-Flupenthixol ^c	400 ± 25	0.52	1.0	900
Fluphenazine ^c	575 ± 25	0.36	2.5	3,000
Haloperidol ^c	850 ± 150	0.42	10.0	3,000
Chlorpromazine ^c	$2,000 \pm 100$	0.38	20.0	7,000
Spiperone ^c	$3,250 \pm 750$	0.33	2.5	3,500

^a IC_{50} value was defined as that drug concentration required to inhibit 50% of specific binding. All specific binding was defined by the inhibition plateau produced by 500 nM apomorphine, occurring at 55% of the total $[^3\text{H}]\text{ADTN}$ binding (see Fig. 6). Some neuroleptics produced two clearly separate phases of inhibition of $[^3\text{H}]\text{ADTN}$ binding. Specific binding to the high affinity site for these neuroleptics was defined by the plateau of the first phase of inhibition occurring at 85% of the total $[^3\text{H}]\text{ADTN}$ binding (see Fig. 6). Specific binding to the low affinity site for neuroleptics was defined as the binding between the first plateau of inhibition and the final plateau of inhibition occurring at 55% of the total binding.

^b Mean $\pm$ SEM.

^c Competition curves with two clearly separate phases.

In the calf, the densities of the D_3 sites and the high affinity neuroleptic sites were approximately 160 to 170 fmol/mg of protein and 39 to 55 fmol/mg of protein, respectively (as measured by $[^3\text{H}]\text{ADTN}$ or $[^3\text{H}]\text{apomorphine}$). Compared to the rat, the calf had about 150% more D_3 sites and 34% fewer high affinity neuroleptic sites.

No sites with high affinity for neuroleptics were detected in the calf or rat using $[^3\text{H}]\text{dopamine}$. As shown in Table VI, the B_{max} for all displaceable $[^3\text{H}]\text{dopamine}$ binding was equal to the B_{max} for the D_3 site (as detected using $[^3\text{H}]\text{apomorphine}$ or $[^3\text{H}]\text{ADTN}$). The B_{max} for all specific displaceable binding of $[^3\text{H}]\text{ADTN}$ and $[^3\text{H}]\text{apomorphine}$, in contrast, was equal to the sum of B_{max}

values for the D_3 site and the high affinity neuroleptic site. Control experiments were performed using rat cerebellum or boiled striatum. In these tissues, no specific binding sites could be detected by Scatchard analyses using $[^3\text{H}]\text{apomorphine}$, $[^3\text{H}]\text{ADTN}$, or $[^3\text{H}]\text{dopamine}$.

Discussion

The present study demonstrates that $[^3\text{H}]\text{ADTN}$ binds to two distinct dopaminergic sites and provides an approach for the selective labeling of these sites individually. One site labeled by $[^3\text{H}]\text{ADTN}$ appears to be the D_3 site, characterized by its high affinity for dopaminergic catecholamines and its low affinity for neuroleptics (previously identified using $[^3\text{H}]\text{dopamine}$ (List et al., 1980)). The second site labeled by $[^3\text{H}]\text{ADTN}$ is a site with high affinity for both dopaminergic catecholamines and neuroleptics. A similar site has been identified recently using $[^3\text{H}]\text{apomorphine}$ and $[^3\text{H}]\text{domperidone}$ (Sokoloff et al., 1980a, b). Our present work indicates that, under our assay conditions, $[^3\text{H}]\text{ADTN}$ and $[^3\text{H}]\text{apomorphine}$ label both of the above described sites, while at low (nanomolar) concentrations, $[^3\text{H}]\text{dopamine}$ labels only D_3 sites.

Catecholamine dopamine agonists (including dopamine, apomorphine, and NPA) in rat striatum and calf and human caudate competed for the same amount of total $[^3\text{H}]\text{ADTN}$ binding. In the rat and human brain, ADTN itself competed for somewhat more $[^3\text{H}]\text{ADTN}$ binding (5 to 10% more) than other drugs. As these sites had no affinity for any of the dopamine-related drugs tested, it appears that $[^3\text{H}]\text{ADTN}$ may label some non-dopaminergic radioligand-specific sites (a phenomenon previously observed using $[^3\text{H}]\text{apomorphine}$ (Leysen and Gommeren, 1981) and $[^3\text{H}]\text{spiperone}$ (Howlett et al., 1979)). Neuroleptics competed for the same amount of $[^3\text{H}]\text{ADTN}$ binding as the dopaminergic catecholamine agonists. Many neuroleptics, however, (e.g., chlorpromazine, haloperidol, spiperone, metoclopramide, sulpiride, and domperidone) showed two distinctly separate phases to their competition. IC_{50} values of the low affinity neuroleptic competition were similar to neuroleptic IC_{50} values for the D_3 site (List et al., 1980). In order to determine whether $[^3\text{H}]\text{ADTN}$ could label these same

Figure 7. A representative Scatchard analysis of [^3H]ADTN binding (range, 0.2 to 6.0 nM) to homogenates of rat striatum and calf caudate. All displaceable binding of [^3H]ADTN was that displaceable by 1 μM apomorphine. The D_3 component was the binding of [^3H]ADTN which occurred in the presence of 30 nM spiperone (to occlude high affinity sites for neuroleptics) but which was displaceable by 1 μM apomorphine. The high affinity neuroleptic component was the binding of [^3H]ADTN which was displaceable by 30 nM spiperone. B (abscissa), specific [^3H]ADTN bound (femtomoles per mg of protein); B/F (ordinate), bound/free.

TABLE VI
Components of [^3H]ADTN, [^3H]apomorphine, and [^3H]dopamine binding

All values are the means $\pm$ SE for two to five independent experiments.

Species	Ligand	All Specific Sites ^a		Low Affinity Neuroleptic Site (D_3) ^b		High Affinity Neuroleptic Site ^c	
		B_{max}	K_D	B_{max}	K_D	B_{max}	K_D
		fmol/mg protein	nM	fmol/mg protein	nM	fmol/mg protein	nM
Rat	[^3H]ADTN	146 $\pm$ 10	2.0 $\pm$ 0.2	76 $\pm$ 8	2.1 $\pm$ 0.4	77 $\pm$ 8	2.0 $\pm$ 0.4
	[^3H]Apomorphine	112 $\pm$ 11	1.6 $\pm$ 0.2	73 $\pm$ 13	1.9 $\pm$ 0.5	62 $\pm$ 13	4.2 $\pm$ 0.6
	[^3H]Dopamine	68 $\pm$ 8	2.5 $\pm$ 0.5			ND ^d	
Calf	[^3H]ADTN	207 $\pm$ 67	1.5 $\pm$ 0.1	162 $\pm$ 50	1.8 $\pm$ 0.4	55 $\pm$ 12	1.4 $\pm$ 0.6
	[^3H]Apomorphine	222 $\pm$ 50	2.1 $\pm$ 0.8	171 $\pm$ 53	1.8 $\pm$ 0.6	39 $\pm$ 12	2.0 $\pm$ 0.4
	[^3H]Dopamine	185 $\pm$ 29	2.1 $\pm$ 0.9			ND	
Human	[^3H]ADTN	84 $\pm$ 3	1.1 $\pm$ 0.1				
	[^3H]Dopamine	63 $\pm$ 10	2.2 $\pm$ 0.4				

^a The heading "All Specific Sites" refers to the binding inhibited by 1 μM apomorphine (using [^3H]dopamine or [^3H]ADTN) or 1 μM dopamine (using [^3H]apomorphine or [^3H]dopamine).

^b Selective binding to D_3 sites was defined as that binding which occurred in the presence of 30 nM spiperone and which was inhibited by 1 μM apomorphine or 1 μM dopamine.

^c Selective binding to these sites was defined as that binding which was inhibited by 30 nM spiperone.

^d ND, not detectable.

previously described D_3 sites selectively, binding assays were carried out in the presence of 30 nM spiperone. (Spiperone at this concentration was found to inhibit [^3H]ADTN binding to the sites with high affinity for neuroleptics but not the sites with low affinity for neuroleptics.) As shown in Figure 8, in the presence of 30 nM

spiperone, drug IC_{50} values against [^3H]ADTN correlated well with those previously observed at the D_3 site.

Neuroleptic competition against [^3H]apomorphine binding in rat striatum produced distinctly separate high affinity and low affinity phases, similar to those observed using [^3H]ADTN (Fig. 4). In contrast, [^3H]dopamine at

Figure 8. The IC_{50} values for various drugs competing in rat striatum against the binding of either $[^3\text{H}]\text{ADTN}$ (in the presence of 30 nM spiperone; data from Table I) or $[^3\text{H}]\text{dopamine}$ (data from List et al., 1980) are similar. The correlation coefficient is 0.96.

low concentrations (i.e., less than 1 nM) appeared to label only D_3 sites as indicated by the absence of a high affinity phase for neuroleptic competition (Fig. 4).

In order to measure the number of D_3 sites and high affinity neuroleptic sites labeled by $[^3\text{H}]\text{ADTN}$, $[^3\text{H}]\text{apomorphine}$, and $[^3\text{H}]\text{dopamine}$, Scatchard analyses were done under conditions which allowed selective labeling of these two types of sites in the calf and rat brain (Fig. 7; Table VI). $[^3\text{H}]\text{ADTN}$ and $[^3\text{H}]\text{apomorphine}$ detected both the D_3 sites and the sites with high affinity for neuroleptics. The B_{max} values of each site were similar regardless of which ligand was used, further supporting the suggestion that both ligands were labeling the same two sites. The B_{max} value obtained for all specific displaceable binding was equal to the sum of the densities for the D_3 and the high affinity neuroleptic sites, indicating that no other sites were being labeled significantly. $[^3\text{H}]\text{Dopamine}$, on the other hand, failed to detect the high affinity neuroleptic sites, and the B_{max} of all specific binding of $[^3\text{H}]\text{dopamine}$ was equal to the density for the D_3 site (as measured by $[^3\text{H}]\text{ADTN}$ or $[^3\text{H}]\text{apomorphine}$). The present observations are supported by a report of Hamblin and Creese (1980), suggesting that, in calf caudate, $[^3\text{H}]\text{dopamine}$ labels a subset of the sites labeled by $[^3\text{H}]\text{apomorphine}$ or $[^3\text{H}]\text{ADTN}$.

We have demonstrated previously (List et al., 1980) that the D_3 site labeled by $[^3\text{H}]\text{dopamine}$ was distributed only in dopaminergic areas of the brain and was heat labile. These observations supported the dopaminergic nature of this binding site. Similarly, the dopaminergic nature of the second site, labeled by $[^3\text{H}]\text{ADTN}$ and $[^3\text{H}]\text{apomorphine}$, with high affinity for neuroleptics, was supported by the failure of the present study to detect this site in preparations of boiled rat striatum and in the non-dopaminergic brain area, cerebellum.

The number of D_3 sites compared to the number of high affinity neuroleptic sites (labeled by $[^3\text{H}]\text{ADTN}$ or

$[^3\text{H}]\text{apomorphine}$) varied considerably between species. In terms of actual densities, the rat striatum contained almost equal amounts of both sites, while in the calf, D_3 sites were 3-fold higher in density than the high affinity neuroleptic site. In neuroleptic competition experiments against 0.8 to 0.9 nM $[^3\text{H}]\text{ADTN}$, the high affinity phase comprised approximately 50% of the specific binding in the rat, 25% of the specific binding in the human, and only about 17% of the specific binding in the calf. Several studies have found considerable differences in the $[^3\text{H}]\text{ADTN}$ and $[^3\text{H}]\text{apomorphine}$ IC_{50} values between the calf and the rat (Creese and Snyder, 1978; Creese et al., 1978, 1979; Davis et al., 1980). The present results suggest that this difference may be due in part to the greater contribution of the high affinity neuroleptic site to $[^3\text{H}]\text{apomorphine}$ and $[^3\text{H}]\text{ADTN}$ binding in the rat compared to the calf. The low proportion of these high affinity neuroleptic sites in the calf caudate is consistent with previous work from a number of laboratories which failed to note the presence of these sites and which reported drug IC_{50} values against the binding of $[^3\text{H}]\text{apomorphine}$ and $[^3\text{H}]\text{ADTN}$ in the calf under nonselective conditions, which agree well with IC_{50} values obtained at the D_3 site (Burt et al., 1975, 1976; Seeman et al., 1976b, 1979; Thal et al., 1978; Cross et al., 1979; Titeler et al., 1979). If the D_3 site is labeled selectively by $[^3\text{H}]\text{dopamine}$ (List et al., 1980) or by $[^3\text{H}]\text{ADTN}$ (in the presence of 30 nM spiperone; this study), the binding characteristics of these ligands correlate well between species.

Differences in the assay conditions also may play a role in directing $[^3\text{H}]\text{ADTN}$ or $[^3\text{H}]\text{apomorphine}$ primarily to the D_3 site or the high affinity neuroleptic site. While $[^3\text{H}]\text{ADTN}$ under our assay conditions had an equal affinity for both sites in the rat, $[^3\text{H}]\text{apomorphine}$ had a 2-fold higher affinity for D_3 . Consistent with these results, we have found previously that, using low concentrations (0.5 nM) of $[^3\text{H}]\text{apomorphine}$, binding was predominantly to D_3 sites (Titeler et al., 1979). Leysen (1979), using higher concentrations of $[^3\text{H}]\text{apomorphine}$ and a slightly different buffer, appeared to label predominantly the sites with high affinity for neuroleptics.

Large differences in the effects of brain lesions on the binding of ^3H -catecholamine agonists have been observed between laboratories (Creese et al., 1978; Nagy et al., 1978; Creese and Snyder, 1979; Leysen, 1979; Weinreich and Seeman, 1980). These differences, however, may be due to the fact that these ligands can label multiple sites. Under conditions where the D_3 site was primarily labeled, kainic acid lesions of the striatum had no effect, while 6-OH-dopamine lesions of nigrostriatal dopamine neurons reduced binding, indicating that at least some of the D_3 sites are presynaptic (Nagy et al., 1978; Weinreich and Seeman, 1980; Sokoloff et al., 1980b). In contrast, Leysen (1979) found that $[^3\text{H}]\text{apomorphine}$ binding was unchanged after 6-OH-dopamine lesions and was reduced by kainic acid. In the study by Leysen (1979), however, as previously discussed, $[^3\text{H}]\text{apomorphine}$ appeared to label a considerable number of the high affinity sites for neuroleptics. Consistent with these findings, using procedures which selectively label the high affinity neuroleptic sites with $[^3\text{H}]\text{apomorphine}$, Sokoloff et al. (1980b)

have shown that these sites were increased or unaffected by 6-OH-dopamine lesions and were reduced significantly by kainic acid. These results suggest that some of the high affinity sites for neuroleptics are postsynaptic.

The biological relevance of a number of the now described dopaminergic sites and their relationship to dopaminergic pharmacology is still unclear. Dopamine receptor terminology often classifies receptors into two types. D₁ dopamine receptors have been defined by their ability to stimulate adenylate cyclase activity while D₂ dopamine receptors have been defined as those which do not stimulate cyclase activity (Kebabian and Calne, 1979). D₁ at present fulfills some, but not all, of the criteria for a bona fide dopamine receptor. The dopamine-stimulated cyclase has a pharmacology which is dopaminergic in nature, it is distributed in dopaminergic regions of the brain, and it can be specifically and saturably labeled using *cis*-[³H]flupenthixol or [³H]piflutixol (Clement-Cormier and Robison, 1977; Hyttel, 1978, 1981). The affinities or potencies of drugs at the dopamine-stimulated cyclase, however, do not correlate with the pharmacology of any yet known dopamine-mediated behavior or biological effect.

D₂ dopamine receptors, which can be labeled by ³H-butyrophenone neuroleptics, such as [³H]spiperone, [³H]haloperidol, and [³H]domperidone, or ³H-ergots, such as [³H]dihydroergocryptine, show a dopaminergic pharmacology quite distinct from that of D₁. In contrast to D₁, the affinities of neuroleptics for D₂ correspond well to their potencies as antagonists for various dopamine-mediated events, such as apomorphine-induced stereotypy, turning, and emesis as well as dopamine inhibition of prolactin secretion in the pituitary (Snyder et al., 1975; Leysen et al., 1977; Caron et al., 1978; Seeman, 1980). The affinity of neuroleptics for the D₂ receptor (labeled by [³H]haloperidol and [³H]spiperone) also has been shown to correlate well with the potencies of these drugs in treating schizophrenia (Seeman et al., 1976a). There is evidence now indicating that (in the pituitary at least) there are D₂ dopamine receptors with nanomolar affinity for dopamine and neuroleptics which produce their biological responses via a dopamine-inhibited cyclase (Munemura et al., 1980; Cote et al., 1981; Meunier and Labrie, 1981).

The D₂ receptor sites, labeled by ³H-butyrophenones and ³H-ergots, usually have been typified by a high affinity for potent neuroleptics (with IC₅₀ values of 1 to 30 nM) and a low affinity for dopaminergic catecholamines, such as dopamine, apomorphine, and ADTN (with IC₅₀ values of 100 to 10,000 nM) (Seeman et al., 1975, 1976a, b; Burt et al., 1976; Titeler et al., 1977; List and Seeman, 1980). As one of the sites labeled by [³H]ADTN in our present study had a high affinity for neuroleptics as well as for dopaminergic catecholamines, one might expect ³H-butyrophenones to label this site as well (specifically by low (nanomolar) concentrations of [³H]spiperone, [³H]domperidone, or [³H]haloperidol, since these neuroleptics had low IC₅₀ values (ranging from 0.15 to 3 nM) for this [³H]ADTN binding site in the present study). Present work has indeed suggested, and more recent work has demonstrated, that [³H]spiperone and [³H]haloperidol label not only sites with high affinity

for neuroleptics and low affinity for dopaminergic catecholamines but also sites with high affinity for both neuroleptics and dopaminergic catecholamines (Titeler et al., 1978b; Creese et al., 1979; Howlett and Nahorski, 1980; Sokoloff et al., 1980b). These two sites labeled by ³H-butyrophenones may be interconvertible through the use of ions and GTP (Sokoloff et al., 1980b; Sibley et al., 1981). Based on this evidence and extrapolating from the studies of other adenylate cyclase-linked receptors, Sibley et al. (1981) have suggested that the two apparent binding sites labeled by ³H-butyrophenones may represent high affinity and low affinity agonist states of the D₂ receptor. Drug IC₅₀ values for the site with high affinity for neuroleptics, labeled in the present study by [³H]ADTN, are very similar to drug IC₅₀ values for the site with high affinity for dopaminergic catecholamines and neuroleptics labeled by the ³H-butyrophenones. Our present results thus support the suggestion by Sokoloff et al. (1980b) that [³H]apomorphine and [³H]ADTN (present study) label a site that also is labeled by the ³H-neuroleptics (i.e., a site with high affinity for both dopaminergic catecholamines and neuroleptics).

Thus, the two sites in the brain labeled by [³H]apomorphine and [³H]ADTN appear to be the D₃ site and possibly a high affinity state of the D₂ receptor.

Some D₃ sites, as discussed, appear to have a presynaptic location on dopamine nerve terminals, which makes them a possible candidate for the dopamine autoreceptor (Carlsson, 1977; Titeler et al., 1978a). While the binding properties of the D₃ site are well known (high affinity for dopamine, low affinity for neuroleptics), the pharmacology of the autoreceptor is not very well characterized. If the D₃ site is the dopamine autoreceptor, one would expect the autoreceptor to be highly sensitive to dopamine agonists (this has been well demonstrated; see Carlsson, 1977; Skirboll et al., 1979) but not very sensitive to the action of neuroleptics (this has been observed by some workers (see Iversen et al., 1976) but not by others (see Strömbom, 1981)).

The present results support the observations of others that [³H]ADTN and [³H]apomorphine label multiple binding sites. Our results indicate that, under our assay conditions, [³H]ADTN and [³H]apomorphine label two sets of dopaminergic binding sites: the D₃ site and a site with high affinity for both neuroleptics and dopamine agonists. The selectivity and affinity of [³H]ADTN and [³H]apomorphine for various binding sites are dependent upon the tissue and the assay conditions. The labeling of different amounts of these various sites in different species and laboratories may account for the variations in the binding characteristics observed for these ligands. Using selective binding techniques, as described in this paper, the D₃ and the high affinity neuroleptic sites can, using [³H]ADTN or [³H]apomorphine, be labeled and studied individually.

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