Biochemical Pharmacology, Vol. 32, No. 19, pp. 2873–2880, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

PHARMACOLOGY OF HIGH-AFFINITY BINDING OF [^{3}H](±)2-AMINO-6,7-DIHYDROXY-1,2,3,4-TETRAHYDRONAPHTHALENE (ADTN) TO BOVINE CAUDATE NUCLEUS TISSUE

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(*Received* 15 *October* 1982; *accepted* 23 *February* 1983)

Abstract—High-affinity binding of [^{3}H](±)2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene ([^{3}H]-ADTN) was improved by use of a subcellular fraction (P_4) of tissue obtained from calf brain. The highest concentration of binding sites was found in caudate nucleus which was evaluated extensively. Binding of 0.5 nM [^{3}H]-ADTN was optimal at 25° and pH 7.5 to 8.0 when a cation-free medium containing antioxidants was used and was 82–87% displaceable ("specific"). The $T_{\frac{1}{2}}$ for association was 20 min, and for dissociation 38 min, under these conditions. Analysis of association–dissociation kinetics and of ligand saturation isotherms revealed an apparent affinity (K_d) of 1–2 nM and a binding maximum (B_{max}) of 422 fmoles/mg protein. The process proved to be reversible by, and monophasically competitive with, several potent dopamine agonists, with Hill constants close to unity. Stereoselectivity was found with eight isomer-pairs. Binding of [^{3}H]-ADTN was selective for 3,4-dihydroxyphenethylamines and 10,11-dihydroxyaporphines with potent dopamine-agonist actions, but not for adrenergic catecholamines or other catechols, or blockers of dopamine uptake. Dopamine antagonists competed more weakly and in poor correspondence with their *in vivo* activities. There was a close correspondence between IC_{50} values obtained for fifty of the agents tested with both [^{3}H]-ADTN and [^{3}H]-(−)apomorphine (r and slope > 0.9), supporting impressions of the structure–activity characteristics of dopamine agonist binding sites based on prior studies with [^{3}H]-apomorphine.

The use of dopamine (DA) agonists and antagonists to label binding sites proposed to be associated with DA receptors recently has been thoroughly reviewed [1]. Biochemical and pharmacological characterization of the high-affinity binding of tritiated DA agonists as ligands usually has employed [^{3}H]-DA or [^{3}H]-(−)apomorphine (APO). The latter is a complex, rigid structural analog of DA which permits extensive chemical and stereochemical modifications with which to explore characteristics of the presumptive receptive surface [2–4]. Nevertheless, while APO has many properties of a DA agonist, it also has mixed agonist/antagonist properties and has been reported to interact with sites defined by the binding of tritiated neuroleptic DA antagonists [1, 4]. The aminotetralins, such as 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN), are an additional class of rigid DA analogs containing a chiral carbon atom proposed as a possible probe of DA receptors. ADTN has the properties of a DA agonist [5], with most of its activity residing in the (+)-enantiomer [6].

Tritiated racemic ADTN has been available for use in binding assays, but only recently in sufficiently high specific radioactivity to permit exploration of high-affinity binding in the nanomolar range. Earlier use of generally labeled [^{3}H]-ADTN of lower specific activity yielded a potentially misleading impression of binding, with affinity in the micromolar range at which DA transport sites and other tissue components might be encountered [7]. With more intensely labeled [^{3}H]-ADTN, recent reports have evaluated the binding of this DA agonist in tissues of the basal ganglia of several species, including rat [8–12] and human [13], as well as in beef caudate tissue [14, 15]. In the studies cited, there has been much less biochemical and pharmacological characterization of high-affinity binding of [^{3}H]-ADTN than with other ligands. Work with calf caudate tissue has been especially limited and includes several unexpected and inconsistent observations. These include atypically low affinities for DA agonists (10–200 nM), higher affinity for some DA antagonists than for agonists, and higher affinity for β-adrenergic agonists than for some DA agonists–often under unfavorable conditions of assay involving a low proportion of specific binding (*ca.* 30%) and a low density of binding sites (68–100 fmoles/mg protein) [14, 15].

We now report application of methods found previously to yield a high proportion of specific binding of [^{3}H]-APO [2, 16] to a calf caudate membrane preparation to evaluate high-affinity binding of [^{3}H]-ADTN under well characterized conditions of

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assay. An extensive structure–activity evaluation is provided by over eighty compounds, including rigid aporphine analogs of DA not previously available, and direct comparisons between potency rankings with [^{3}H]-ADTN and [^{3}H]-APO.

MATERIALS AND METHODS

Methods

Tissue preparation. Tissue of the caudate nucleus was prepared as described previously [2, 16]. Thus, caudate tissue was dissected from brain tissue (*ca.* 3.5 g/brain) of calves (not treated with meat tenderizing proteases) within 15 min of death, placed on dry ice, stored overnight at −70°, and prepared for binding assays on the following day. Tissue was thawed to 4° in 0.32 M sucrose (5 ml/g tissue), homogenized using a glass Potter–Elvehjem homogenizer and Teflon pestle (clearance, 0.25 mm) with ten up-and-down strokes at 600 rpm and centrifuged at 1000 *g* for 10 min. The resulting nuclear and debris pellet was discarded, and the supernatant fraction (S_1) was collected and immediately centrifuged at 20,000 *g* for 20 min at 4° in a Sorvall RC2-B; the supernatant fraction (S_2) was discarded and the resulting pellet (P_2) was resuspended in 100 vol. of purified water at 4°, immediately mixed in a Fisher Vortex Genie mixer, allowed to stand for 15 min at 4°, remixed, further disrupted with a Brinkmann Polytron (PT-10 probe) set at 6 for 30 sec, and centrifuged at 8000 *g* for 20 min. The supernatant fraction (S_3) was saved, and the loose intermediate layer of suspended tissue ("buffy coat") above a mitochondria-rich pellet was resuspended using a small volume of S_3; both S_3 and "buffy coat" were combined and centrifuged at 38,000 *g* for 20 min. the final pellet obtained (P_4) was resuspended in pure water (1 ml/g wet weight caudate) and frozen at −20° in 1-ml portions to be used in the receptor assay within 4 months.

An aliquot of the frozen P_4 fraction was thawed in a 4° water bath immediately before each assay and washed with 10 ml of ice-cold 50 mM Tris·HCl (pH 7.5) buffer by mixing twice for 15 sec with the Vortex mixer set at 6. This mixture was centrifuged at 48,000 *g* for 10 min at 4°; the supernatant fraction was discarded, and the same washing and centrifugation steps were repeated. The washed pellet, thus prepared, was resuspended in 5 vol. of *assay buffer* containing the following: 50 mM Tris·HCl, 5 mM Na_2EDTA, 6 mM Na-ascorbate, and 12.5 μM nialamide, at a final pH of 7.5. The tissue preparation was further disrupted with the Polytron set at 6 for 10 sec and transferred with a disposable polypropylene pipette to the assay tubes. These final tissue samples yielded 0.2 to 0.4 mg protein/assay, as measured by a commercial dye-absorption method (Bio-Rad Protein Assay, Richmond, CA) using bovine serum γ-globulin as standards.

Binding assay. The standard radioligand binding assay was similar to methods described previously [2, 16, 17]. Thus, it was performed in 13 × 100 mm borosilicate glass culture tubes (VWR Scientific, Boston, MA), cleaned with an air stream filtered through spun glass. Until the incubation was started, assay tubes were kept on ice. The following components were added to the tubes with polypropylene-tipped automatically dispensing pipettes to provide a final incubation volume of 1 ml: (a) 0.25 ml assay buffer (see above), with or without test compound added; (b) 0.25 ml assay buffer, with or without 40 μM unlabeled apomorphine (APO) for [^{3}H]-ADTN binding or 40 μM unlabeled ADTN for the [^{3}H]-APO binding as "blanks," to yield final concentrations of 10 μM; (c) 0.25 ml of 2 nM [^{3}H]-ADTN or [^{3}H]-APO in the assay buffer to yield a final concentration of 0.5 nM of radioactive ligand (for kinetic analyses, the concentrations ranged from 0.1 to 20 nM); and (d) 0.25 ml of membrane preparation (200–400 μg protein) suspended in the assay buffer added immediately prior to incubation.

The incubation was initiated by adding the membrane suspension and transferring the tubes from the ice bath to a slowly agitated 25° water bath for 60 min. Incubation was terminated by the addition of 5 ml of ice-cold 50 mM Tris–HCl (pH 7.5) buffer to each tube followed by rapid filtration (< 2 sec) over Whatman glass fiber filters (GF/B, 25 mm diameter) held in Millipore manifolds under constant vacuum. The filters were washed twice more with 5 ml of the Tris buffer and then transferred to polypropylene scintillation counting vials, shaken thoroughly for 15 min, allowed to equilibrate at 4° for 4 hr, and counted for tritium in a TriCarb liquid scintillation sepctrometer (Packard Instruments, Downers Grove, IL) at *ca.* 40% efficiency.

Data were computed as fmoles [^{3}H]-ADTN or [^{3}H]-APO specifically bound per mg protein ± S.D.; S.D. was consistently less than 5% of the mean. Kinetic analyses were carried out by computing association and dissociation constants, as well as from saturation isotherms evaluated by the method of Scatchard and depicted graphically according to Eadie and Hofstee, and the slopes of inhibition functions were evaluated by the method of Hill–all as described in Boeynaems and Dumont [18].

Materials

(±)[5,8-^{3}H]2-Amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene ([^{3}H]-ADTN; sp. act. 41.5 Ci/mmole) and (−)[8,9-^{3}H]apomorphine·HCl ([^{3}H]-APO; 38–40 Ci/mmole) were obtained from the New England Nuclear Corp. (Boston, MA) at greater than 95% purity as confirmed by thin-layer chromatography [17] before and after representative assays. Both were received in an aqueous phosphate buffer (10 mM, pH 3.0) containing 5% (vol.) ethanol and stored light-free at 4° under argon gas until divided into aliquots. Aliquots (2.5 ml) were prepared (1.2 mM) in the same buffer and stored at 4° under nitrogen in capped polypropylene vials until used in the binding assays, typically within 30 days. Authentic (−)apomorphine·HCl·$\frac{1}{2}H_2O$ (APO) was obtained from McFarlan-Smith (Edinburgh, Scotland) and a recently prepared and purified sample of authentic unlabeled (±)5,6-ADTN was obtained from Burroughs Wellcome (Research Triangle, NC); (±)5,6-ADTN was from Research Biochemicals (Wayland, MA).

Aporphine compounds were prepared and purified by Dr. John L. Neumeyer and his colleagues at the Department of Medicinal Chemistry, North-

eastern University (Boston, MA). Neuroleptic agents were donated by the following sources: (+) and (−)butaclamol (Ayerst Laboratories, New York, NY); clozapine (Sandoz Laboratories, Basel, Switzerland); *cis*- and *trans*-flupenthixol (H. Lundbeck & Co., Copenhagen, Denmark); fluphenazine·HCl (E. R. Squibb & Sons, New Brunswick, NJ); haloperidol (McNeil Laboratories, Fort Washington, PA); chlorpromazine·HCl and trifluoperazine·HCl [as well as (+)amphetamine·H_2SO_4 and (+)-*p*-hydroxyamphetamine·H_2SO_4] (Smith, Kline & French, Philadelphia, PA); loxapine·HCl (Lederele Laboratories, Wayne, NJ); molindone·HCl (Endo Laboratories, Wilmington, DL); perphenazine Schering Corp., Bloomfield, NJ); sulpiride (S.E.S.I. Laboratories, Ile de France); and thiothixene (Roerig, New York, NY). Other drug gifts were: buspirone·HCl (Mead Johnson, Evansville, IN); the (+)- and (−)-enantiomers of α-methyldopamine·HCl and cocaine·HCl (Merck Laboratories, West Point, PA); (+)norepinephrine·HCl (Sterling-Winthrop Laboratories, Rensselaer, NY); *N,N*-dimethyldopamine and *O.O'*-dibenzoylapomorphine·HCl (Dr. Robert J. Borgman, Arnar-Stone Laboratories, McGraw Park, IL); carbamazepine (Geigy Pharmaceuticals, Ardsley, NY); desipramine·HCl (Merrell-Dow Laboratories, Cincinnati, OH); metergoline (Farmitalia Carlo Erba, Milan, Italy); nomifensine (Hoechst Laboratories, Somerville, NJ); pergolide (Eli Lilly & Co., Indianapolis, IN); piribedil (Servier Laboratories, Orleans, France); and (±)3-(3-hydroxyphenyl)*N*-*n*-propylpiperidine (3-PPP; Dr. Arvid Carlsson, University of Göteborg, Sweden). All other drugs and chemicals were obtained from commercial sources in the highest available purity.

RESULTS

General characteristerics of [^{3}H]-ADTN binding to calf caudate tissue

Evaluation of several fractions of bovine caudate nucleus tissue indicated the highest proportion of displaceable or "specific" binding of [^{3}H]-ADTN (85%), a high apparent affinity (1.3 nM), and the highest apparent binding site density per mg protein (422 fmoles) in the P_4 fraction (Table 1), as found previously with [^{3}H]-APO [2, 16]. Thus, the binding of [^{3}H]-ADTN to the P_4 subcellular fraction of calf caudate tissue was evaluated further. When the assay employed 0.5 nM [^{3}H]-ADTN as ligand and 10 μM APO was used as the "blank" to define specific binding, the proportion of specific to total binding of tritium was typically 82–87%. Binding was found to be linearly dependent on protein ($r > 0.90$) between amounts (per ml) of 0.1 to 1 mg; protein concentrations were typically between 0.2 to 0.4 mg per assay sample. There was no specific binding when tissue was omitted. When pH was varied in the range of 6 to 9, a pH optimum appeared at 7.5 to 8.0; pH 7.5 was routinely used to reduce the risk of oxidation of the catechol ligand [17]. When the temperature was varied between 0° and 37° (at constant pH = 7.5), it was found that 25° was the optimum incubation temperature, as determined by the specific counts bound per mg protein. When 0.5 to 1.0 nM [^{3}H]-ADTN was employed as the ligand concentration, less than 5% of the total counts in the medium were bound to the membrane preparation, indicating "Zone-A" conditions of binding [18] that justified omitting corrections for small changes in free concentration of ligand (F) in kinetic analyses. Thin-layer chromatography indicated that over 90% of [^{3}H]-ADTN was recovered after incubation under standard assay conditions [17].

The selection of 10 μM APO as an appropriate blank is supported by the finding that the amount of tritium bound after incubation of 0.5 nM [^{3}H]-ADTN for 60 min at 25° was virtually identical after excesses of unlabeled APO, ADTN, DA, or (+)butaclamol. Thus, the proportion of displaceable ("specific") to total binding in the presence of 10 μM or 100 μM APO, or 10 μM ADTN, DA, or (+)butaclamol was 81–82% in each case. Moreover, the effects of a broad range of concentrations of unlabeled APO against the binding of 0.5 nM [^{3}H]-ADTN demonstrated apparently monophasic competition over three orders of magnitude (IC_{50}

Table 1. [^{3}H]-ADTN binding kinetics in calf caudate membrane fractions*

Tissue fraction	K_d (nM)	B_{max} (fmole/mg protein)	% Specific binding
Crude pellet	1.0	91	71
P_2 pellet	1.7	225	74
P_2 (after water and polytron)	1.4	340	76
P_4 (standard perparation)	1.3	422	85

* The crude pellet was prepared from homogenate, centrifuged at 48,000 *g* for 20 min twice, and washed with buffer without discarding subcellular fractions, much as reported in previous studies by other laboratories. The P_2 was made from S_1 without osmotic shock or rehomogenization with the Polytron; the third fraction was P_2 subjected to water at 0° for 10 min and disruption in the Polytron set at 6 for 30 sec. Apparent K_d and B_{max} values were calculated from linear transformations of kinetic data, as in Fig. 1, with six concentrations of [^{3}H]-ADTN ranging from 0.25 to 5.0 nM, using 10 μM APO as blank; Scatchard analysis was highly linear ($r = 0.99$); each point represents a mean of three separate determinations with S.D. < ± 5% of the mean. The data indicate apparent enrichment of binding sites with each succeeding step, with a corresponding improvement in the proportion of saturable ("specific") binding.

= 4.9 nM); the Hill constant [18] of this process (n_H was 1.09. Up to 85% of total binding was displaced by 500 nM, and there was no further inhibition up to 100 μM. This observation further supports the choice of 10 μM APO to define the blank for all subsequent pharmacologic experiments.

The binding of 0.5 nM [^{3}H]-ADTN to P_4 fractions prepared from calf brain regions revealed very weak binding in frontal cortex, hypothalamus, occipital cortex, cerebellum, and brainstem-medulla, ranging from 2 to 6% of that in caudate tissue. The proportion of specific binding ranged from 4 to 17%.

Kinetic characteristics of [^{3}H]-ADTN binding to fractions of calf caudate

The binding of low concentrations of [^{3}H]-ADTN to calf caudate (P_4) revealed a saturable, high-affinity component of binding which appeared to follow monophasic saturation (Fig. 1). A linear ($r = 0.99$) transformation of these data (Fig. 1, inset) revealed estimates of apparent binding site density (B_{max}) of 422 fmoles/mg protein and affinity (K_d) of 1.27 nM. The time-course of binding of [^{3}H]-ADTN to P_4 was also followed past equilibrium over 180 min at 25°. Binding equilibrated approximately by 60 min, after which there was little (<5%) additional binding for the next 100 min. To some tubes, excess unlabeled APO (10 μM) was introduced after 60 min, and the assays were followed over the next 2 hr to approximate equilibrium of dissociation of 80% of the bound [^{3}H]-ADTN at 180 min. Conversion of these data to linear logarithmic expressions allowed computation of kinetic constants for association and dissociation [18]. Values of $T_{\frac{1}{2}}$ for association (19.6 min) and dissociation (38.3 min) were computed. The ratio of the computed association (0.0346) and dissociation (0.0181) constants yielded a value of apparent $K_d = 1.91$ nM, in good agreement with the value of K_d (1.27 nM) obtained by linear analysis of a saturation isotherm (see Fig. 1).

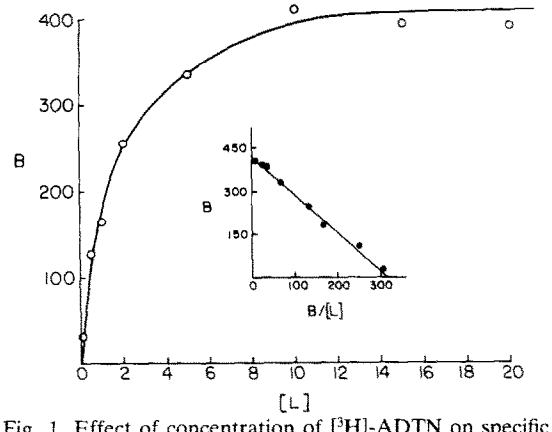

Fig. 1. Effect of concentration of [^{3}H]-ADTN on specific binding. Data were obtained with bovine caudate (P_4 fraction) by incubating at 25° for 60 min, with and without 10 μM APO to define a blank—all as described in Methods. Data represent mean values of three independent experiments, each analyzed at all points in triplicate (S.E.M. < ±5% of means). Inset: Eadie–Hofstee [18] linearized plot of the saturation isotherm indicating apparent B_{max} = 422 fmoles/mg protein and K_d = 1.3 nM.

Pharmacologic characteristics of [^{3}H]-ADTN binding

Determinations of IC_{50} values for eighty-one compounds are summarized in Table 2. For comparison, results with [^{3}H]-APO under similar conditions for most of the same compounds also are shown (Table 2). The first seven compounds are dopamine (DA) agonists, including DA, (−)apomorphine, pergolide, and (±)ADTN, all of which had IC_{50}s values of less than 5 nM. Among the most potent competitors for [^{3}H]-ADTN (and [^{3}H]-APO) binding were both the mono- and di-*N*-methylated congeners of DA (epinine and *N*,*N*-dimethyldopamine) with IC_{50}s values of 2.8 and 6.5 nM, respectively, as well as the levo-enantiomer and the racemate of 10,11-dihydroxy-*N*-propylnoraporphine (NPA), which yielded IC_{50}s values of 2.0 and 3.5 nM respectively (compounds 2 and 4, Table 2).

A comparison of (−) vs (+)NPA (IC_{50} = 2 vs 70 nM; compounds 2 and 19a, Table 2) indicates that the (−)stereoisomer of NPA was the more active form with respect to competition with the high-affinity binding of [^{3}H]-ADTN; its Hill constant was 1.00. In addition to NPA, stereoselectivity of binding was demonstrated by several other pairs of compounds: (−) vs (+)2,10,11-trihydroxyaporphine (compounds 11 and 48: IC_{50} = 11 vs 1600 nM); (−) vs (±)11-hydroxy-*N*-*n*-propylnoraporphine (compounds 9 and 16: IC_{50} = 7 vs 40 nM); (+) vs (−) 1,2-dihydroxyaporphine (compounds 21 and 44: IC_{50} = 145 and 1140 nM); (−) vs (+)norepinephrine (compounds 18 and 32: IC_{50} = 45 vs 371 nM); (+) vs (−)butaclamol (compounds 20 and 68: IC_{50} = 74 vs 80,000 nM); and (−) vs (+) α-methyldopamine (compounds 36 and 43: IC_{50} = 488 vs 1130 nM); in addition, the pharmacologically more active geometric *cis*-isomer of flupenthixol was more potent (compounds 15 and 52: 30 vs 5000 nM; Table 2).

(−)Norepinephrine and (−)epinephrine were 10- to 100-fold less potent than the more potent dopaminergic agonists tested. The relatively low-affinity binding of (−)norepinephrine (compound 18: IC_{50} = 45 nM), (−)epinephrine (compound 36a: 500 nM), (±)isoproterenol (compound 55: > 10,000 nM), and the enantiomers of α-methyldopamine (compounds 36 and 43: 488 and 1130 nM) also suggests that substituents (e.g. OH or CH_3) on either the α or β carbons of the ethylamine side chain of phenethylamines interfere with [^{3}H]-ADTN binding, and that the binding site defined with a low concentration of [^{3}H]-ADTN (0.5 nM) is neither an adrenergic (α or β) receptor nor a non-specific catechol-binding site. Other catechols containing a carboxyl group were also weak antagonists (DOPA, DOPAC; IC_{50} > 10,000 nM; compounds 57 and 58, Table 2). Moreover, the DA-uptake blockers, (+)amphetamine, nomifensine, and cocaine, had little effect on the binding of [^{3}H-ADTN (Table 2).

A free 10,11-dihydroxy configuration yielded potent inhibition of [^{3}H]-ADTN binding among aporphines (compounds 2, 4, 6, 7, 10, and 11: IC_{50} < 12 nM; Table 2). Comparison of monohydroxylated compounds suggests that the 11-OH may be the more important site for binding within the catechol moiety of 10,11-dihydroxyaporphines. Consider: (−)11-OH-aporphine vs (±)10-OH-aporphine

Table 2. Potency of binding of eighty-one agents against [^{3}H]ADTN and [^{3}H]APO*

	Compound	IC_{50} (nM) [^{3}H]-ADTN	IC_{50} (nM) [^{3}H]-APO
1.	(±)6,7-Dihydroxy-2-aminotetralin (6,7-ADTN)	1.0	1.8
2.	(−)10,11-Dihydroxy-*N*-*n*-propylnoraporphine · HCl [(−)NPA]	2.0	2.5
2a.	Pergolide	2.0	
3.	*N*-Methyldopamine (*N*-Me-DA, epinine)	2.8	3.4
4.	(±)10,11-Dihydroxy-*N*-*n*-propylnoraporphine · HI [(±)NPA]	3.5	5.0
5.	Dopamine (DA)	3.7	4.5
6.	(−)Apomorphine · HCl	4.9	1.0
7.	(−)2,10,11-Trihydroxy-*N*-*n*-propylnoraporphine · HBr	5.5	25
8.	*N*,*N*-Dimethyldopamine (*N*,*N*-DiMe-DA)	6.5	13
9.	(−)11-Hydroxy-*N*-*n*-propylnoraporphine · HBr	7.0	5.4
10.	(−) 2,10,11-Trihydroxy-*N*-ethylnoraporphine · HBr	10	25
11.	(−)2,10,11-Trihydroxyaporphine · HBr [(−)TA]	11	10
12.	(−)11-Hydroxyaporphine · HBr	12	7.8
13.	(−)Norapomorphine · HBr	13	14.4
14.	(−)2,10,11-Trihydroxy-*N*-allylnoraporphine · HBr	28	24
15.	*cis*-Flupenthixol	30	260
16.	(±)11-Hydroxy-*N*-*n*-propylnoraporphine · HBr	40	36
17.	(−)2,10,11-Trihydroxynoraporphine · HBr	44	30
18.	(−)Norepinephrine [(−)NE]	45	50
19.	(−)10,11-Dihydroxy-*N*-hydroxyethylnoraporphine · HCl	60	25
19a.	(+)10,11-Dihydroxy-*N*-*n*-propylnoraporphine · HCl [(+)NPA]	70	
20.	(+)Butaclamol	74	230
20a.	Metergoline	80	
20b.	(−)2,11-Dihydroxyaporphine · HBr	114	
21.	(+)1,2-Dihydroxyaporphine · HI	145	124
22.	(±)10-Hydroxyaporphine · HBr	150	112
23.	(−)2,10-Dihydroxyaporphine · HBr	184	295
24.	Haloperidol	223	630
25.	(+)Bulbocapnine (1,2-methylenedioxy-10-methoxy-11-hydroxyaporphine)	230	70
26.	(−)10,11-[Carboxy-methylenedioxy]aporphine	262	143
26a.	(±)3-[3-Hydroxyphenyl]*N*-*n*-propylpiperidine (3-PPP)	273	
27.	(−)Morphothebaine · HCl (2,11-dihydroxy-10-methoxyaporphine)	316	204
27a.	Thiothixene	323	
28.	Trifluoperazine · HCl	335	580
29.	(−)10,11-Methylenedioxy-*N*-*n*-propylnoraporphine · HCl (MDO-NPA)	342	850
30.	Fluphenazine · HCl	346	770
31.	(−)Pukateine (1,2-methylenedioxy-11-hydroxyaporphine)	371	70
32.	(+)Norepinephrine [(+)NE]	371	590
33.	Perphenazine	377	800
34.	Chlorpromazine · HCl	385	1,250
35.	(−)2,11.Dihydroxy-10-methoxy-*N*-*n*-propylnoraporphine · HCl	420	1,030
35a.	(−)*N*-Chloroethylnorapomorphine · HCl (NCA)	468	
36.	(−)α-Methyldopamine [(−)αMeDa]	488	280
36a.	(−)Epinephrine	500	
37.	(±)10-Hydroxy-*N*-*n*-propylnoraporphine · HBr	516	700
37a.	(−)10,11[Heptylidine-2-dioxy]-*N*-propylnoraporphine · HCl	548	
37b.	Loxapine · HCl	*ca.* 560	
38.	(−)2-Hydroxy-10,11-methylenedioxy-*N*-*n*-propylnoraporphine · HCl	586	236
39.	(−)2-Hydroxy-10,11-methylenedioxyaporphine · HCl	627	583
40.	(−)10,11-[Methylethyl-methylenedioxy]-*N*-*n*-propylnoraporphine · HCl	647	1,381
41.	(±)Aporphine · HCl	664	367
42.	(−)10,11-Methylenedioxyaporphine · HCl	899	1,310
43.	(+)α-Methyldopamine	1,130	1,800
44.	(−)1,2-Dihydroxyaporphine · HI	1,140	750
45.	(−)Apocodeine · HCl (10-methoxy-11-hydroxyaporphine)	1,351	2,400
46.	(−)10,11-[Dimethyl-methylenedioxy]-aporphine · HCl	1,359	2,179
47.	(−)10,11-Diacetoxy-*N*-chloroethylnoraporphine · HCl	1,600	1,060
48.	(+)2,10,11-Trihydroxyaporphine · HBr	1,600	*ca.* 5,000
48a.	(±)5,6-Dihydroxy-2-aminotetralin · HBr (5,6-ADTN)	2,600	
49.	(±)8-Hydroxy-*N*-*n*-propylnoraporphine · HI	4,000	*ca.* 10,000
50.	(−)8-Nitro-10,11-methylenedioxy-*N*-*n*-propylnoraporphine · HCl	4,491	6,500
51.	(−)*N*-Chloroethylnorapocodeine · HCl	*ca.* 5,000	
52.	*trans*-Flupenthixol	*ca.* 5,000	*ca.* 50,000
53.	Molindone · HCl	*ca.* 6,000	
54.	(−)2,10-Dimethoxy-11-hydroxyaporphine · HCl	*ca.* 7,200	
55.	(±)Isoproterenol · HCl	>10,000	>10,000
56.	(+)*p*-Hydroxyamphetamine · H_2SO_4	>10,000	

Table 2 (*continued*). Potency of binding of eighty-one agents against [^{3}H]ADTN and [^{3}H]APO*

	Compound	IC_{50} (nM) [^{3}H]-ADTN	[^{3}H]-APO
57.	L-3,4-Dihydroxyphenylalanine (L-DOPA)	>10,000	>10,000
58.	3,4-Dihydroxyphenylacetic acid (DOPAC)	>10,000	>10,000
59.	Piribedil	>10,000	>10,000
60.	(−)*O,O'*-Dibenzoylapomorphine · HCl	>10,000	>10,000
61.	(+)Amphetamine · H_2SO_4	>10,000	
62.	Nomifensine	>10,000	
63.	Cocaine · HCl	>10,000	
64.	Clozapine	>10,000	
65.	(−)*N*-Carbethoxymethyl-norapomorphine · HCl	*ca.* 13,000	
66.	Buspirone · HCl	*ca.* 15,000	
67.	(−)2,10,11-Trihydroxy-*N*-carbethoxymethylnoraporphine · HCl	*ca.* 16,000	
68.	(−)Butaclamol	*ca.* 80,000	>10,000
69.	(±)Sulpiride	*ca.* 100,000	
70.	Carbamazepine	>100,000	

* Binding assays were carried out as described in Methods with 0.5 nM [^{3}H]-ADTN for 60 min at 25°, with and without 10 μM APO as blank. All test agents were incubated in triplicate, typically with four to six concentrations in the region of IC_{50}, which was computed from log-probit analysis (S.E.M. was within ± 10% of each mean tabulated). Compounds 1–50 were plotted in Fig. 2 (lettered compounds or those after number 50 were not included in Fig. 2 as data were incomplete, or the IC_{50} exceeded 5000 nM).

(compounds 12 vs 22: 12 vs 150 nM) and (±)11-OH- vs (±)10-OH-*N*-propylnoraporphine (compounds 16 and 37: 40 vs 516 nM). Nevertheless, the presence of an 11-OH group *per se* on aporphines was not adequate for potent interaction since the presence of a 10-methoxy moiety or other ring-substituents (such as the 2-OH or 1,2-methylenedioxy moieties on the A-ring) resulted in 11-hydroxylated compounds that competed for [^{3}H]-ADTN binding relatively weakly [notably, the (−)isomers of morphothebaine, pukateine, and apocodeine; compounds 27, 31 and 45: IC_{50} = 316, 371, and 1351 nM respectively]. Other dihydroxyaporphines were weaker competitors than 10,11-catecholaporphines. Examples include (+) and (−)1,2,di-OH-aporphine, (−)2,11-di-OH-aporphine, (−)2,10-di-OH-aporphine, and (−)2,11-di-OH-10-MeO-*N*-propylnoraporphine (compounds 21, 44, 20b, 23 and 35: IC_{50} = 145, 1140, 114, 184 and 420 nM respectively). Addition of a 2-OH group to the aporphine system tended to reduce the affinity of 10,11-catecholaporphines (e.g. compounds 7 vs 2, and 11 vs 6: IC_{50} = 5.5 vs 2 nM and 11 vs 4.9 nM respectively), and that of 11-monohydroxyaporphine (compounds 12 vs 20b: 12 vs 114 nM). Addition of *N*-alkyl substituents to 2,10,11-trihydroxyaporphines altered affinity in the order: *n*-propyl > ethyl ≥ methyl > allyl > hydrogen ≥ carbethoxymethyl (compounds 7, 10, 11, 14, 17, and 67: IC_{50} = 5.5, 10, 11, 28, 44, and 16,000 nM respectively; all compounds refer to Table 2).

The neuroleptic, dopamine receptor blocking agents or atypical antipsychotic agents ranked: *cis*-flupenthixol > (+)butaclamol > haloperidol > thiothixene > trifluoperazine > fluphenazine > perphenazine > chlorpromazine > loxapine ≥ molidone > clozapine > sulpiride, with IC_{50}s values of 30, 74, 223, 323, 335, 346, 377, 385, 560, 6000, > 10,000, and *ca.* 100,000 nM respectively (compounds 15, 20, 24, 27a, 28, 30, 33, 34, 37b, 53, 64 and 69; Table 2). Thus, even the more potent DA antagonists displaced [^{3}H]-ADTN at approximately 1/10 to 1/50 the potency of DA agonists. In addition, sulpiride and clozapine were virtually inactive (IC_{50} > 10,000 nM; Table 2).

The congruence of the pharmacologic characteristics of high-affinity binding of [^{3}H]-ADTN and [^{3}H]-APO, based on data from Table 2, is demonstrated by plotting data for the fifty compounds that were evaluated under similar conditions with both ligands and have IC_{50} values with respect to [^{3}H]-ADTN of below 5000 nM (Fig. 2). The results revealed a close correlation (linear regression of the logarithms of the IC_{50} data yielded $r = 0.94$ [$P < 0.001$] and slope = 0.89).

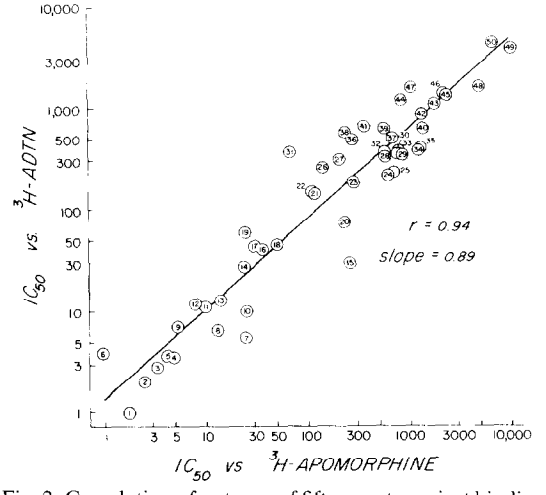

Fig. 2. Correlation of potency of fifty agents against binding of [^{3}H-ADTN and [^{3}H-apomorphine. Data correspond to the numbered compounds in Table 2. Pearson correlation factor (r = 0.94) and the slope function (0.89) were computed by linear regression analysis by the method of least-squares, using the logarithm$_{10}$ for all IC_{50} values.

DISCUSSION

Previous use of [3H]-ADTN as a radioligand to label binding sites proposed to be associated with DA receptors in the central nervous system has led to some results that are inconsistent with expectations arising from the use of other DA-agonist ligands [1–4], as was mentioned in the beginning of the paper. For example, the IC_{50} (or K_i) for DA vs [3H]-ADTN (nM binding is expected to be low, but ranged from 3 [12] or 13 nM [16], to 80 [14, 210 [19], 300 nM [8, 9], and 1 mM [7]. Some DA agonists, notable DA itself, were reportedly weaker in competition with [3H]-ADTN than some neuroleptics with crude homogenates of rat striatum [8, 9, 12]. With calf caudate homogenates, the adrenergic agonist epinephrine was reported to have greater affinity than the potent DA agonist apomorphine (APO) [15]. Rank-ordering of DA-agonist potency has also varied; it was: ADTN > epinine > DA > APO in one laboratory [15] and ADTN > DA > APO in another [14]–both in agreement with the results in Table 2—but ADTN ≥ APO > DA in others [8, 9, 12], and ADTN > APO > DA ≥ epinine in still another [11]. There does seem to be agreement that the (+)-isomer of ADTN is more potent than (−)ADTN in da-agonist binding assays [15, 16, 19], as expected [1, 3, 12]. Such variable results with [3H]-ADTN binding reported in the past may reflect dissimilarities in the conditions of assay, including possible species, or developmental differences, the presence or absence of exogenous cations, the use of a high assay temperature (> 30°) [8–10, 12, 14] that may alter DA-agonist binding sites or increase decomposition of such ligands [17], the presence of a relatively low proportion of displaceable (specific) binding [8–11, 13–15], the use of relatively high concentrations of ligand [7], or very short incubation times [12].

The inconsistencies in previous studies with [3H]-ADTN binding and the limited pharmacologic characterization available suggested that further investigations were warranted. In the work reported here, the characteristics of binding of [3H]-ADTN to the P_4 fraction of calf caudate included a high proportion of specific total counts bound (82–85%), linear dependency on the amount of tissue, and strong dependence on temperature and pH. Since physiologic concentrations of Na^+ or a physiologic mixture of cations decreased [3H]-ADTN binding (data not shown) and since complex effects of cations on DA-agonist binding have been reported previously [20, 21], we carried out the present study without adding exogenous cations. We have also reported previously that the presence of an antioxidant (ascorbic acid) and EDTA is required for a high proportion of specific binding and to minimize the chemical degradation of DA-agonist ligands [2, 16, 17].

While we found a decrease of [3H]-ADTN binding on assays conducted at 37°, the significance of this change is not clear since a loss of DA-agonist binding sites in bovine caudate [16, 22] as well as increased degradation of the ligand [17] may occur. To complicate matters further, Bacapoulos [20] has reported recently that binding of [3H]-DA to *rat* striatal homogenates can be *increased* by *pre*-incubation of tissue at 37°. In contrast, we found that warming bovine P_4 caudate tissue during preincubation at 37° for an hour decreased binding of 0.5 nM [3H]-ADTN. This loss was prevented by including a millimolar concentration of DA during the preincubation at 37° (unpublished observation). This effect of DA is evidently not due to preservation of the ligand since [3H]-ADTN was introduced only in a second incubation for binding at 25° after washing repeatedly to remove exogenously added DA. Perhaps DA prevents thermal denaturation of a specific agonist site, analogous to the stabilizing effect of substrates on enzymes [23]; we also found a similar protective effect of DA against loss of binding sites by incubation with a protease. Alternatively, DA may hold a crucial membrane component in a physicochemical state favorable to the high-affinity binding of an agonistligand, as DA has been reported to enhance the binding of other DA agonists [20, 24]; we have also found that preincubation with DA can increase the binding of [3H]-ADTN to calf caudate P_4 fractions (unpublished observations). The apparent differences in responses of bovine and rat striatum to heating remain to be elucidated.

The pharmacology of [3H]-ADTN binding at low nanomolar concentrations (Table 2) appears to follow characteristics expected of a DA-agonist receptor site, and resembles the high-affinity binding of [3H]-APO (Fig. 2) as described previously [1–4, 16, 17]. Thus, adrenergic catecholamines were much weaker than DA agonists and the presence of a catechol moiety *per se* was not sufficient to assure high-affinity binding. Moreover, blockers of DA uptake (Table 2) were not effective against the high-affinity binding of [3H]-ADTN, even though the latter may interact at DA transport sites at higher (μM) concentrations [7, 24]. Neuroleptics were not only weak antagonists of [3H]-ADTN binding (Table 2), but their rank-order of potency bore little correspondence to their *in vivo* potencies [25], in contrast to results with tritiated butyrophenone ligands [1, 26]. In addition, the seven neuroleptics included in Fig. 2 were consistently more potent versus [3H]-ADTN than [3H]-APO (3.35-fold ± 0.92, S.E.M.) for uncertain reasons, although their IC_{50}s values against the two DA-agonist ligands were closely correlated (logarithmic $r = 0.89$). In a separate analysis of the thirty-five aporphines presented in Fig. 2, they showed no significant preference for the aporphine ligand, [3H]-APO, over [3H]-ADTN (logarithmic $r = 0.95$, slope = 0.87 for [3H]-ADTN vs [3H]-APO—further suggesting that low concentrations of both ligands (≤ 1 nM) bind at sites that may reflect the DA-structural homologies of both ligands.

The structure–activity relationships indicated in the findings summarized in Table 2 are described in detail in Results. Briefly, 3,4-catechol phenethylamines without side-chain carbon-substitution (i.e. DA and its *N*-alkyl analogs), as well as *N*-alkylated (−)10,11-dihydroxyaporphines, were especially potent and most are reported to be DA agonists by behavioral tests or stimulation of adenylate cyclase [1, 5]. The 11-hydroxy position of aporphines, corresponding to the meta-OH of DA, appears to be

a particularly critical site of interaction with the binding site, as we have reported previously [2–4, 27]. Stereochemical selectivity of binding in competition with [^{3}H]-ADTN was found with several isomeric pairs (Table 2). The more potent isomer of each pair was consistently the pharmacologically more active form in cases in which this is known [e.g. (−)catecholaporphines, (+)butaclamol, *cis*-flupenthixol, and (−)norepinephrine] [2–4]. The results obtained by the present structure–activity evaluation are not only very similar to results obtained with [^{3}H]-apomorphine [2] (Table 2, Fig. 2) and [^{3}H]-DA [1], but also provide further support for molecular characterizations of DA-agonist binding sites described elsewhere [1–4, 27–29].

The present observations with [^{3}H]-ADTN and [^{3}H]-APO describe a site with high affinity for DA (nM) and low affinity for neuroleptics (0.1 to 1.0 μM). These sites have generally been referred to as "D-3" sites, and their biological role is not known [1, 21, 30, 31]. It has also been suggested that such sites, as well as some sites characterized by a high (nM) affinity for ^{3}H-antagonists of DA, can be detected by [^{3}H]-ADTN [30] and have been referred to as "D-4" sites [1] or "D-2-high" sites [21, 30].

In conclusion, the results obtained indicate that the high-affinity binding of [^{3}H]-ADTN can be evaluated under improved conditions that yield a high proportion of saturable and displaceable ("specific") high-affinity binding with kinetic characteristics and molecular pharmacologic characteristics expected of a DA-agonist binding site that may indicate a class of DA-agonist receptors in mammalian brain tissue. Both [^{3}H]-ADTN and [^{3}H]-APO appear to be useful rigid analogs of DA with which to explore molecular pharmacologic characteristics of DA receptors. It would appear to be an appropriate and timely step now to prepare and evaluate [^{3}H]-(+)ADTN or the (+)-enantiomers of its *N*-alkyl substituted analogs as ligands which might have even further specificity for DA receptors than [^{3}H]-(±)ADTN.

Acknowledgements—This work was supported, in part, by USPHS NIMH Research Grant MH-34006 and Research Career Award MH-47370 (to Dr. Baldessarini), NINCDS Grant NS-15439 and Northeastern University Distinguished Professor Award (to Dr. Neumeyer); and an award from the Scottish Rite Schizophrenia Foundation and from an NIH Biomedical Support Grant RR-05484 (to Dr. Arana). Technical assistance was provided by Ms. Nora Kula and Ms. Sabrina Popp. The manuscript was prepared by Mrs. Mila Cason.

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