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BINDING OF [^{3}H]ADTN TO RAT STRIATAL MEMBRANES

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Abstract—The conformationally restricted dopamine analogue ADTN binds in a specific saturable manner to rat striatal membranes. Analysis of the data suggests a single binding site. Binding of [^{3}H]ADTN is displaced by a wide range of dopamine agonists and antagonists (both typical and atypical). The potency of ADTN derivatives to displace [^{3}H]ADTN correlates well with their activity as agonists in other tests both *in vivo* and *in vitro*.

Brain dopamine receptors have been extensively studied by radioligand binding techniques. Several ligands have been used including antagonists such as [^{3}H]-*cis*-flupenthixol [1], [^{3}H]spiroperidol [2], [^{3}H]haloperidol [3], [^{3}H]pimozide [4], [^{3}H]domperidone [5] and [^{3}H]sulpiride [6]. Less commonly, agonists including [^{3}H]apomorphine [7], [^{3}H]dopamine [3] or [^{3}H]ADTN (2-amino-6,7-dihydroxy-1,2,-3,4-tetrahydronaphthalene) [8] have been employed. It is important that both ^{3}H-agonists and ^{3}H-antagonists should be utilised in binding studies, primarily to confirm if binding is occurring to the same membrane component.

Unique problems occur in the use of each ligand. Spiroperidol (spiperone) has been considered a 'drug of choice' for dopamine receptor labelling [2] since it displays a very high percentage of specific binding, a high affinity and a low dissociation rate, but this neuroleptic will also label 5-hydroxytryptamine receptors [9]. Similarly, some authors [10] consider that [^{3}H]-*cis*-flupenthixol does not display adequate selectivity for dopamine receptors.

While antagonist binding may be readily observed, some groups have encountered difficulties with agonist binding; for example, that binding is not readily displaceable by neuroleptics [11]. Unlike antagonists, the choice of agonist ligands is somewhat limited. Dopamine itself is metabolically unstable. Apomorphine has the advantage of a low dissociation rate from the receptor [7, 12] but, in some cases is only a partial agonist [13] while 6-propylnorapomorphine is less active than dopamine [14]. ADTN is a conformationally restricted analogue of dopamine (with the receptor preferring configuration) but is not more bulky than dopamine and enjoys the advantage of being a full agonist which is consistently equipotent with dopamine in all tests [15]. ADTN is also more stable metabolically than dopamine (ADTN is not a substrate for MAO) but unfortunately, a rapid dissociation rate remains a problem.

The dopaminergic nature of ADTN activity has been extensively investigated using electrophysiological [16, 17], behavioural [18], and biochemical [19] techniques. It is therefore surprising that doubt has been cast on the suitability of [^{3}H]ADTN as a dopamine receptor ligand [20]. In view of this, we wish to confirm and extend previous observations on the dopaminergic nature of [^{3}H]ADTN binding in rat striatum.

MATERIALS AND METHODS

Materials. [^{3}H]ADTN (35 Ci/mmole) was obtained from New England Nuclear (Boston, MA). Purity was checked at regular intervals by TLC on silica gel plates with butanol: acetic acid: water (25:4:10) as solvent. *cis*-Flupenthixol and trans-flupenthixol were gifts from Lundbeck (Copenhagen, Denmark); sulpiride from Ravizza S.P.A. (Milan, Italy); tiapride and sultopride from Chemitechna (Petersfield, U.K.); and fluphenazine from E. R. Squibb (Twickenham, U.K.)

Preparation. Crude striatal membranes were prepared from rat striata (Male Wistar, 250–300 g) suspended in 0.32 M sucrose in 5 mM Tris–HCl, pH 6.5 (at 10% w/v) and homogenised using 12 strokes at 900 rpm of a loose-fitting Teflon–glass homogeniser. The nuclear fraction (P_1) was removed by centrifugation (800 *g*, 10 min), the supernatant saved and the pellet washed once with a further 10 vol. buffer. The supernatants were pooled and a mitochondrial pellet (P_2) prepared by centrifugation (17,000 *g*, 20 min). The membranes in this pellet were lysed by homogenisation, using a tight pestle, in 5 mM Tris–HCl (pH 8) and recovered after centrifugation (45,000 *g*, 20 min). The pellet finally obtained was resuspended in 50 mM Tris–Krebs (pH 7.4) and membranes were then stored in liquid nitrogen for up to one week.

Binding assay. Membranes containing 150–200 μg protein were preincubated at 37° for 2 min in the presence or absence of 1 μM dopamine or 1 μM (±)-ADTN (which defined specific binding) or of other drugs or vehicles. The incubation proper was initiated by addition of [^{3}H]ADTN (0.4–54 nM for saturation analysis or 5 nM in displacement studies). After 10 min, the tubes were placed on ice for 2 min. to minimise dissociation of [^{3}H]ADTN [21] bound ligands were separated from free by filtration under vacuum over prewashed glass fibre filters (Whatman,

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GF/B), which were then washed with 15 ml ice-cold buffer. Protein on filters was solubilised with 1 ml 2% sodium deodecyl sulphate, 8 ml scintillation fluid added and radioactivity determined by liquid scintillation counting with correction for quenching.

Protein was estimated [22] and results were expressed as fmole [^{3}H]ADTN bound/mg protein.

RESULTS

Binding characteristics. [^{3}H]ADTN binds specifically to rat striatal membranes. Specific binding is saturable in the range 0.4–54 nM. At these concentrations non-specific binding is linear. Figure 1 shows data from a typical experiment.

Specific binding can be defined by 1 μM (±)-ADTN or by 1 μM dopamine and, under the conditions of the assay, constitutes 50–60% of the total binding at 5 nM [^{3}H]ADTN. Binding of [^{3}H]ADTN to the glass fibre filters is unaffected by 1 μM (±)-ADTN or 1 μM dopamine.

The Scatchard plot of the data from saturation experiments is linear and indicates a maximum binding capacity of 221 fmole/mg protein with an affinity constant of 11 nM. Figure 2 shows the plot from a typical experiment. When binding data are transformed using the Hill equation, a good fit is obtained ($r = 0.96$) with a slope of 0.89 (Fig. 3).

Displacement studies. The IC_{50} values for various drugs to displace the binding of [^{3}H]ADTN (5 nM) to striatal membranes are shown in Table 1.

[^{3}H]ADTN displays a marked stereoselectivity towards neuroleptics, thus, (±)-butaclamol is almost 9000 times more active than the (−) enantiomer, and *cis*-flupenthixol has 100 times greater activity than the clinically inactive *trans* isomer. Also, the (+) isomer of ADTN itself is 14 times more potent to displace [^{3}H]ADTN than the (−) isomer.

Dopaminergic agonists are potent displacers of [^{3}H]ADTN being more potent than apomorphine which, in turn, is more potent than dopamine. Of the substituted derivatives of ADTN, *N,N*-di-n-propyl-*iso*-ADTN is a more potent displacer than ADTN itself while *iso*-ADTN (2-amino-5,6-dihydroxy-1,2,3,4-tetrahydronaphthalene) shows reduced activity. The dimethoxy derivative of *iso*-ADTN is of lowest activity.

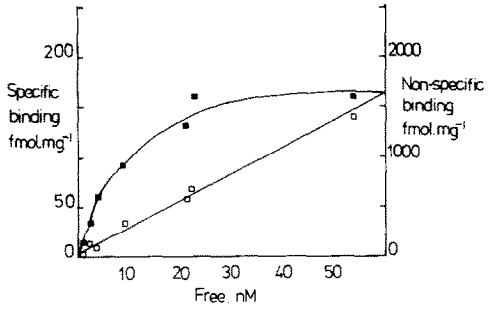

Fig. 1. [^{3}H]ADTN binding to crude striatal membranes; a typical experiment. Specific binding (■) was displaceable by 1 μM (±)-ADTN. Non-specific binding (□) was that occurring in the presence of 1 μM (±)-ADTN. Each point represents triplicate determinations. Left ordinate, [^{3}H]ADTN specifically bound; right ordinate, non-specific binding of [^{3}H]ADTN.

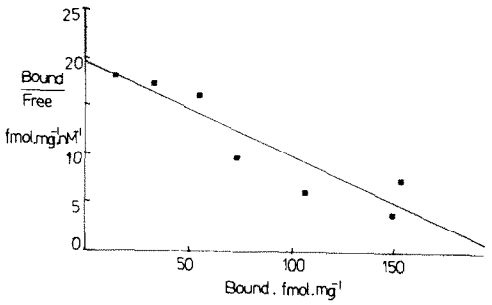

Fig. 2. Scatchard analysis of [^{3}H]ADTN specific binding to crude striatal membranes; a typical experiment. The line of best fit gives values for K_d of 10.7 nM, and B_{max} 211 fmole/mg protein. Abscissa, bound (fmole/mg); ordinate, bound/free (fmole/mg nM^{-1}). Data from 4 separate experiments yield K_d 11 nM and B_{max} 221 fmole/mg protein.

The ergot alkaloid derivatives, lisuride, bromocriptine, pergolide and ergometrine, which have partial agonist activity are of intermediate potency.

[^{3}H]ADTN binding is displaced by neuroleptics of all major classes, most potent being (+)-butaclamol, domperidone, sulpiride, spiroperidol and *cis*-flupenthixol. [^{3}H]ADTN binding appears to be specific for dopaminergic sites since noradrenaline, adrenaline and 5-hydroxytryptamine were inactive as displacers.

DISCUSSION

ADTN is a potent dopamine receptor agonist in several tests of dopamine receptor function, thus ADTN stimulates the dopamine-sensitive adenylate cyclase [19] and has agonist activity in electrophysiological [16] and behavioural [18, 23] models. Our results provide evidence for specific saturable binding of [^{3}H]ADTN to striatal membranes.

Scatchard analysis of the binding suggests a single high affinity site with affinity constant 11 nM and B_{max} 221 fmole/mg protein. The Hill plot of ADTN binding data (Fig. 3) gives a slope close to one suggesting simple first order kinetics. This is comparable with the high affinity binding site for ADTN, in rat striatum, reported by Creese and Snyder [9]

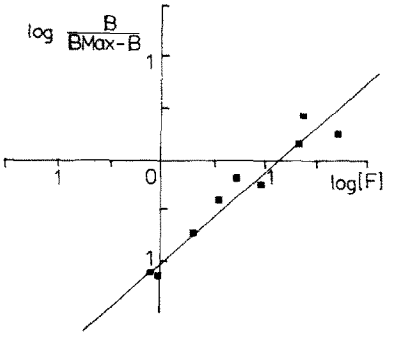

Fig. 3. Hill plot of [^{3}H]ADTN specific binding. A B_{max} of 221 fmole/mg protein (from Scatchard analysis) has been assumed. Slope = 0.89. Abscissa, log concentration of free ligand; ordinate, log bound ligand/B_{max} − bound ligand.

Table 1. Inhibition of [^{3}H]ADTN binding to rat striatal membranes by drugs

Drug*	IC_{50} (nM)†
N,N-di-n-propyl *iso*-ADTN	0.4
(+)-Butaclamol	1.3
(+)-ADTN	1.7
Domperidone	2.1
Sulpiride	2.2
(±)-ADTN	3.6
Apomorphine	7.1
Sultopride	8.8
Dopamine	12.6
Spiroperidol	15.0
Tiapride	18.2
(−)-ADTN	23.0
cis-Flupenthixol	23.0
iso-ADTN	30.0
Lisuride	36.0
Ergometrine	50.0
Bromocriptine	56.0
Molindone	86.0
SKF 38393	149.0
Dimethoxy-*iso*-ADTN	286.0
Haloperidol	468.0
Chloropromazine	557.0
Pergolide	1645.0
trans-Flupenthixol	2143.0
(−)-Butaclamol	11800.0

* (−)-Noradrenaline, (−)-adrenaline and 5-hydroxytryptamine were relatively inactive (IC_{50} > 10,000 nM).

† Concentration of various drugs required to displace 50% of 5 nM [^{3}H]ADTN specific binding to rat striatal membranes. Specific binding was defined by 1 μM (±)-ADTN. Each value represents results from at least 3 experiments, using at least 4 concentrations, with each estimation in triplicate. Standard errors were 5–10% of the mean.

which has a K_d of 6 nM and B_{max} 15 pmole/mg wet wt, and with that detected by Davies *et al.* [24] with K_d 8.8 nM and B_{max} 116 fmole/mg protein. Similarly, a single high affinity site for ADTN has been reported in calf striatum by several groups [8, 25]. These reports, and the present findings, are at variance with the failure to observe specific saturable binding [20] and it is difficult to resolve this anomaly. In particular, the reduction of [^{3}H]ADTN binding by ascorbic acid or EDTA cited [20] is not relevant since these were excluded from the assay buffer in the present experiments, Kaayalp and Neff [26, 27] having previously reported the adverse effects of ascorbate on ^{3}H-agonist binding.

The high affinity ADTN binding site appears to be of a specific dopaminergic nature in view of the potencies of dopamine receptor agonists and antagonists to displace [^{3}H]ADTN compared with noradrenaline, adrenaline or 5-hydroxytryptamine. The order of potency of displacers agrees with previously published data for the agonists, dopamine and apomorphine. With respect to agonists, (±)-ADTN is more potent than apomorphine, which is more potent than dopamine. Of the ADTN derivatives, the dipropyl-*iso* derivative is at least as potent as ADTN, *iso*-ADTN somewhat weaker and the dimethoxy-*iso*-derivative weakest of all. This parallels the relative activities of these drugs in both behavioural tests and the dopamine sensitive cyclase assay [23] with the exception that the methoxy derivative has no activity whatsoever in cyclase or behavioural tests but has a slight activity as a displacer.

The availability of the enantiomers of ADTN allows investigation of the stereospecificity of the dopamine receptor with enantiomers of an agonist. In the present study, (+)-ADTN was 14-fold more active than (−)-ADTN, which agrees with ratios of 7 and 16 previously reported [8, 28] but is substantially less than the 390-fold difference in activity previously reported [24]. A possible explanation for this discrepancy could be that Davis *et al.* [24] used a very high concentration of dopamine (1 mM) to define specific binding compared with 1 μM (±)-ADTN used in the present study. In behavioural studies, involving the stimulation of motor activity by injection of dopamine agonists into the nucleus accumbens, the ratio of activity of (+)-ADTN to (−)-ADTN is 13 [23], although such results cannot be directly compared with binding studies due to complicating factors in the whole animal.

[^{3}H]ADTN was displaced by neuroleptics of all major types. Most potent was (+)-butaclamol, the (−)-isomer being some 9000 times less active; also of high potency were the benzamides, sulpiride, sultopride and tiapride, the butyrophenones spiroperidol and domperidone and the thioxanthene, *cis*-flupenthixol; chlorpromazine and haloperidol were fairly weak displacers of [^{3}H]ADTN, while the activity of the substituted ergots, which behave as partial agonists in the cyclase assay, ranged from fairly potent (lisuride) to fairly weak (pergolide).

With respect to the classical neuroleptics, these results are consistent with previous reports on [^{3}H]ADTN binding. Of particular interest are the effects of the ergots and benzamides, which have not previously been reported with respect to ADTN binding. Thus the benzamides, which have no effect on the stimulation of cyclase by dopamine [29], and the ergots, which have atypical effects on the adenylate cyclase assay [30], are all capable of displacing [^{3}H]ADTN binding.

In binding experiments with [^{3}H]apomorphine or [^{3}H]dopamine agonists often prove to be more potent displacers than antagonists. Similarly, agonists can be less effective than antagonists to displace ^{3}H-antagonist binding. These observations have been cited as evidence for a two-state receptor [3]. However, the profile of displacers of [^{3}H]ADTN does not support such a model since agonist and antagonist potencies are not dissimilar. Also, in view of the fact that [^{3}H]ADTN binding is displaced by a wide range of neuroleptics (typical and atypical), agonists and partial agonists, reputed to label a variety of sites, it is difficult to infer whether ADTN binds to one or more than one class of dopamine receptor.

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