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THE UPTAKE AND RELEASE OF [^{3}H]-2-AMINO-6,7-DIHYDROXY-1,2,3,4-TETRAHYDRONAPHTHALENE (ADTN) BY STRIATAL NERVE TERMINALS

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1 A study has been made of the uptake and release of [G-^{3}H]-2-amino 6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) by crude striatal synaptosomes of the rat.

2 Uptake was rapid, temperature-dependent and could be suppressed by a variety of metabolic inhibitors.

3 The Michaelis-Menten kinetics indicated the presence of two distinct transport systems in the striatum which were of much higher capacity than those found in the cerebellum, which lacks dopaminergic innervation.

4 Uptake of [^{3}H]-ADTN was strongly inhibited by dopamine and the two potent dopamine-uptake inhibitors, benztropine and nomifensine, but only weakly by imipramine and amphetamine (the latter in non-reserpine-treated animals).

5 Accumulated [^{3}H]-ADTN could be released from striatal slices by elevated K^+. A similar release was evoked upon the addition of the ionophore, A23187.

6 The most potent releaser of [^{3}H]-ADTN was (+)-amphetamine. This effect occurred at concentrations inactive against ADTN uptake. The neuroleptic *cis*-flupenthixol produced an inhibition of the spontaneous release.

7 It is concluded that [^{3}H]-ADTN is accumulated preferentially into areas of the rat brain rich in dopamine. The pharmacological specificity of the uptake suggests that it is a good substrate for the dopamine carrier. Following uptake, [^{3}H]-ADTN may be released by K^+ and a calcium ionophore, which raises the possibility that ADTN might act as a false transmitter.

Introduction

A variety of pharmacological studies have shown that the compound 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN), is a potent agonist at mammalian dopamine receptors (Miller, Horn, Iversen & Pinder, 1974; Munday, Poat & Woodruff, 1974; 1976; Elkhawad, Munday, Poat & Woodruff, 1975; Woodruff, McCarthy & Walker, 1976).

Recently, we have shown that ADTN will bind to dopamine-sensitive sites on highly purified rat striatal membranes (Roberts, Woodruff & Poat, 1977).

Bilateral injection of ADTN into the nucleus accumbens of conscious rats causes a long-lasting stimulation of locomotor activity (Elkhawad & Woodruff, 1975). This action of ADTN is believed to be due to dopamine receptor activation, although the unusually long duration of action raises the possibility that other factors (eg. the formation of false transmitters) might be involved.

Evidence available suggests that the dopamine skeleton present in the molecule of ADTN represents the active conformation of dopamine at the receptor site (Woodruff, Watling, Andrews, Poat & McDermed, 1977). Similar suggestions have been made regarding the active conformation of dopamine at the presynaptic uptake site (Horn, 1974), although direct evidence for the latter is lacking.

The present study was carried out in order to investigate the possible uptake of ADTN and the likelihood of its being able to act as a false dopaminergic transmitter.

Methods

Animals

Female Wistar rats (250 to 350 g) were used throughout the study.

Labelled ADTN

[G-^{3}H]-ADTN was prepared by the Radiochemical Centre, Amersham, by the process of catalytic exchange labelling, using 100 Ci tritiated water. The crude material was stored at −140°C until required. Immediately before use, the [^{3}H]-ADTN was purified by one-dimensional thin-layer chromatography (t.l.c.) on cellulose plates with 1-butanol:water:acetic acid (12:5:3 v/v/v) as solvent system. Two main bands were obtained, and the one corresponding to authentic co-chromatographed unlabelled ADTN was scraped off and eluted with ethanol. Following evaporation of the solvent, the labelled ADTN was redissolved in 5 mM tartaric acid. The concentration of [^{3}H]-ADTN was determined by spectrophotofluorimetric assay of the products formed after reaction with fluorescamine (λ_{act} 390 nm, λ_{fluor} 475 nm) (Udenfriend, Stein, Böhlen, Dairman, Leimgruber & Weigele, 1972). A range of unlabelled ADTN standards (2.5 to 20 nmol/ml) was similarly processed. The specific radioactivity of the [^{3}H]-ADTN was found to be approximately 3 mCi/mmol.

Uptake of [^{3}H]-ADTN into crude (P_2 fraction) synaptosomes

Rats were killed by cervical dislocation and pairs of striata rapidly dissected out and homogenized in 5 volumes ice-cold 0.32 M sucrose, with a loose-fitting teflon-glass homogenizer. The homogenate was centrifuged at 800 *g* for 20 min and the supernatant transferred to a clean tube and centrifuged for a further 20 min at 17,500 *g* to obtain the crude synaptosomal pellet (P_2). This was resuspended in a small volume of 0.32 M sucrose. Incubations were carried out in 10 ml stoppered conical flasks and typically, each flask contained 2 ml Krebs-bicarbonate medium with 1 mg/ml ascorbate to inhibit auto-oxidation of the ADTN and 10 μM nialamide. Synaptosomes were added to give a final protein concentration of 0.3 to 0.4 mg/ml and the flasks were preincubated for 2 min at 37°C after gassing with 95% O_2 and 5% CO_2. Incubations were started by the addition of 10 μl of [^{3}H]-ADTN with further gassing of the flasks. Incubations, which were usually for 4 min, were terminated by rapid filtration under reduced pressure through Whatman GF-C glass fibre filters, using a millipore filter assembly. The filters were rapidly washed with 5 ml Krebs-bicarbonate medium and transferred to scintillation vials. Following solubilization with 0.5 ml 12% NaOH in methanol, 10 ml of scintillation fluid (xylene 6.67 ml, Synperonic NXP 3.33 ml, PPO 40 mg and dimethyl POPOP 5 mg) was added and the accumulated radioactivity determined by liquid scintillation spectrometry with automatic correction for quenching, background and counting efficiency in all cases. Incubations were also carried out at 0°C to give control (blank) values which were subtracted from test values.

Release of [^{3}H]-ADTN from striatal slices

Striata were dissected out as described and slices (10 to 20 mg/slice) were cut with a razor blade by hand. Slices were transferred to 2 ml Krebs-bicarbonate medium in conical flasks and after the addition of [^{3}H]-ADTN to give a final concentration of 1.76 μM, were incubated for 20 min under 95% O_2 and 5% CO_2 at 37°C. Each slice was then removed, blotted and transferred through a series of vials containing 3 ml Krebs-bicarbonate medium at 37°C. The vials were gassed upon tissue addition and incubation continued in each vial for 5 minutes. The effect of drugs on the release of [^{3}H]-ADTN was investigated by their inclusion during the fourth incubation period. Radioactivity released into the vials was determined by liquid scintillation counting following the addition of 0.5 ml methanol and 10 ml scintillant. The radioactivity remaining in the tissue at the end of the incubation was also determined.

Results

Time course of [^{3}H]-ADTN uptake and its subcellular localization

Uptake of [^{3}H]-ADTN (10 μM) into the crude striatal nerve-ending preparation was essentially linear for the first 4 min of incubation, achieving tissue levels of approximately 210 pmol/mg protein (Figure 1). Uptake apparently declined with incubation periods longer than 4 min, which might be attributable to diffusion of metabolites into the medium. The subcellular localization of the uptake sites was primarily synaptosomal. This was established by investigation of the uptake of [^{3}H]-ADTN into purified preparations of mitochondria, myelin and synaptosomes. This approach was found to be more satisfactory than by the incubation of tissue homogenates with labelled ADTN and the subsequent application of subcellular fractionation procedures.

Metabolic stability of [^{3}H]-ADTN

ADTN was not thought to be a substrate for mono-

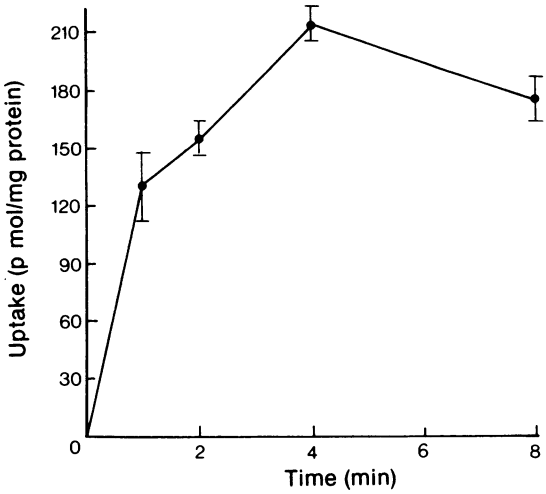

Figure 1 Time course of [^{3}H]-ADTN uptake. Crude synaptosomes were incubated at 37°C with [^{3}H]-ADTN (10 μM) for various periods between zero and 8 minutes. Results are expressed as pmol ADTN accumulated per mg protein. Correction was made for labelled ADTN retained extracellularly. Each point is the mean of 4 independent observations. Vertical lines show s.e. means.

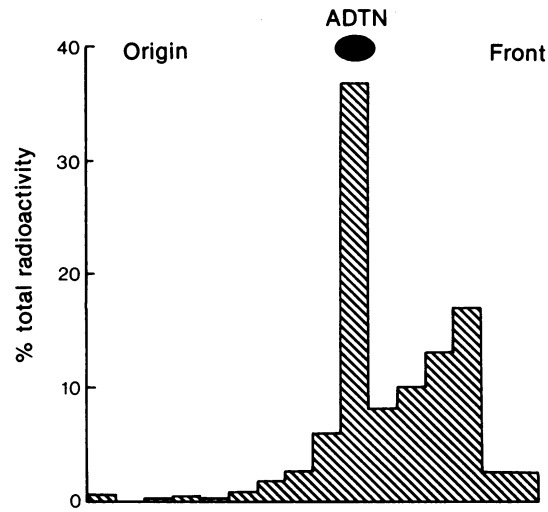

Figure 2 Thin-layer chromatographic separation of [^{3}H]-ADTN from its metabolites produced by incubation of [^{3}H]-ADTN (50 μM) with crude synaptosomes for 20 min at 37°C. The solvent system was as described in the text. One centimetre strips were scraped off the developed chromatogram, from the origin to solvent front, transferred to scintillation vials, eluted with 2 ml ethanol and counted.

amine oxidase but it was considered likely that it could be metabolized by catechol-*O*-methyltransferase (COMT). Although short incubation periods of synaptosomes with [^{3}H]-ADTN 50 μM indicated minimal metabolism, after 20 min t.l.c. of tissue extracts indicated that approximately 45% of the added [^{3}H]-ADTN had been converted to more polar metabolites (Figure 2).

Temperature-dependence and effect of metabolic inhibitors on uptake of [^{3}H]-ADTN

Crude synaptosomes were incubated for 4 min with [^{3}H]-ADTN 0.32 μM over a range of temperatures. Uptake increased up to approx. 37°C and then rapidly decreased (Figure 3). When incubations were carried out in the presence of one of *p*-chloromercuriphenylsulphonate, sodium cyanide, ouabain, or 2,4-dinitrophenol (all at 0.5 mM), uptake was inhibited by at least 50% in all cases.

Kinetic characteristics of [^{3}H]-ADTN uptake

The Michaelis-Menten kinetics were determined by incubation of striatal synaptosomes for 4 min with a wide range of [^{3}H]-ADTN concentrations (0.078 to 10.04 μM). The lines of best fit were plotted by the method of Wilkinson (1961) and two distinct components were resolved (Figure 4). One of these was of high affinity (apparent $K_m = 0.27 \pm 0.10$ μM) and low capacity ($V_{max} = 0.29 \pm 0.05$ nmol.mg^{-1} protein.4 min^{-1}) and, a second of lower affinity (apparent $K_m = 3.06 \pm 0.56$ μM) and high capacity ($V_{max} = 1.02 \pm 0.07$ nmol.mg^{-1} protein.4 min^{-1}).

When uptake was investigated in synaptosomes prepared from the cerebellum, a region of the brain devoid of dopaminergic nerve terminals, a similar pattern emerged with both high and low affinity components (apparent K_ms of 0.28 ± 0.056 μM and 7.53 ± 1.53 μM respectively). The striking difference between the two regions was the much lower capacity of each of the cerebellar systems (V_{max}s = 0.028 ± 0.017 and 0.126 ± 0.013 nmol.mg^{-1} protein.4 min^{-1}, respectively). When uptake was studied with small (approx. 0.5 × 0.5 × 0.1 mm) slices, after a 4 min incubation in the presence of [^{3}H]-ADTN (0.35 μM), a tissue:medium ratio (d.min^{-1}.mg^{-1} tissue/d.min^{-1}.μl^{-1} incubation medium) of 172.4 was attained with the striatum, whilst with the olfactory bulbs, cerebral cortex and medulla, ratios of only 35.7, 20.7 and 8.4 respectively were reached.

Inhibition of [^{3}H]-ADTN uptake by drugs

In an initial screening procedure, crude synaptosomes were incubated with [^{3}H]-ADTN and a high concentration of the potentially inhibitory compound

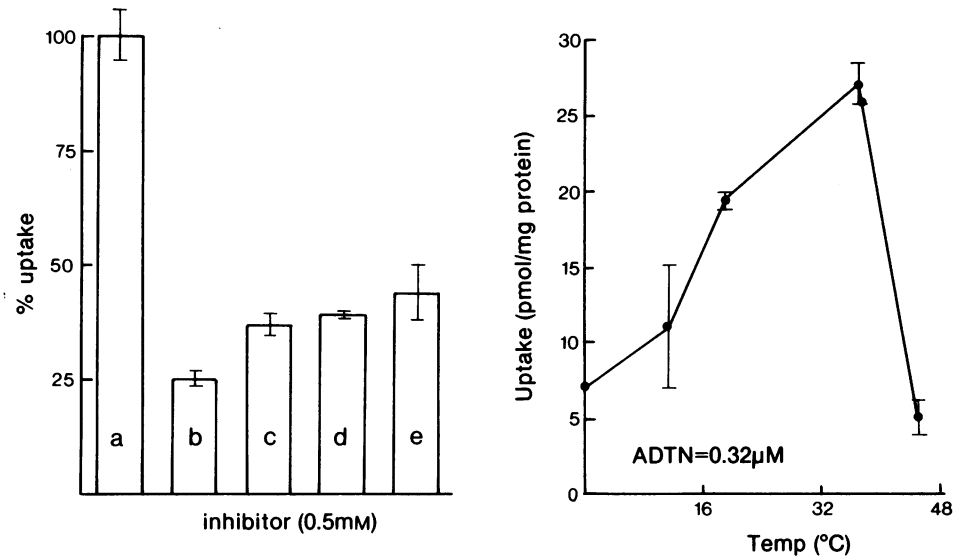

Figure 3 Effect of temperature and metabolic inhibitors on the uptake of [^{3}H]-ADTN. Crude synaptosomes (P_2) were incubated at 37°C for 4 min with [^{3}H]-ADTN (0.32 μM) following a 5 min preincubation with metabolic inhibitors: (a) control, (b) *p*-chloromercuriphenylsulphonate, (c) NaCN, (d) ouabain, and (e) 2,4-dinitrophenol, at a final concentration of 0.5 mM). Results are means of 4 observations. Vertical lines show s.e. means. Temperature-dependence of uptake was studied during a 4 min incubation at temperatures ranging from 0 to 45°C. Results are expressed as pmol/mg protein and are the means of 4 observations (uncorrected for blank value—see text). Vertical lines show s.e. means.

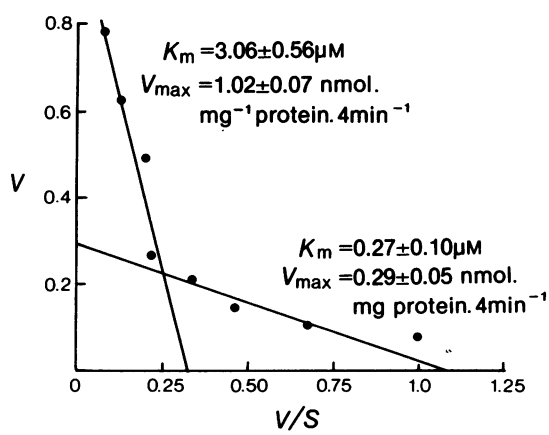

Figure 4 Eadie-Hofstee plot of the uptake of [^{3}H]-ADTN into crude striatal synaptosomes. Synaptosomes were incubated for 4 min at 37°C in the presence of a wide range of [^{3}H]-ADTN concentrations (0.078 to 10.04 μM). The lines of best fit were determined by regression analysis. Each point represents the mean of at least 4 determinations. Uptake (V) is expressed as nmol ADTN accumulated.mg^{-1} protein.4 min^{-1} and concentration (S) as μM.

(usually agents known to influence the transport of dopamine into its nerve terminals). Active compounds were then tested at a number of concentrations and log dose-inhibitor concentration v. % inhibition curves constructed. Inhibitory potencies were expressed as IC_{50} values ie. that concentration of inhibitor producing a 50% inhibition of [^{3}H]-ADTN uptake (Table 1). ADTN and dopamine were found to be roughly equipotent in their abilities to inhibit [^{3}H]-ADTN accumulation. Benztropine and nomifensine which are highly active inhibitors of dopamine uptake (Hunt, Kannengiesser & Raynaud, 1974) were also potent against [^{3}H]-ADTN uptake, with IC_{50}s of approx 200 nM. Noradrenaline (NA) was 5 times less active than dopamine in inhibiting ADTN uptake, whilst the tricyclic antidepressant, imipramine, was almost inactive. Amphetamine was of low potency (IC_{50} = 3350 nM), although its activity was enhanced some 11 fold in synaptosomes prepared from rats which had been pretreated with reserpine in order to deplete the tissue dopamine stores.

A tricyclic imine, N-cyclopropylmethyl-10,11-dihydro-5H-dibenzo-(a,d)-cyclohepten-5-imine (CDCI) has recently been reported to be a specific dopamine uptake inhibitor (Herblin, 1977). However, the drug produced rather inconsistent, but generally weak inhibition in our experiments.

Effect of K^+ and Ca^{2+}-dependency of [^{3}H]-ADTN release

The addition of 50 mM K^+ (substituted for sodium) to the incubation medium, doubled the release of [^{3}H]-ADTN from striatal slices (Figure 5a). The omission of calcium ions and the supplementation of the medium with 10 mM Mg^{2+}, produced a reduction in the spontaneous release. However, the potassium-evoked release was only marginally inhibited. An alternative approach, that of using the carboxylic acid antibiotic ionophore, A23187, which rather selectively influences the movements of calcium (Foreman, Mongar & Gomperts, 1973), resulted in a marked increase in the release of [^{3}H]-ADTN when exposed to low concentrations (5 to 10 μg/ml) of the compound (Figure 5b).

Effects of drugs on the release of [^{3}H]-ADTN

The most potent releaser of [^{3}H]-ADTN was found to be (+)-amphetamine. The effect was dose-dependent and was first apparent at a concentration of 0.1 μM, which was found to be inactive against ADTN uptake (Figure 6). ADTN itself and nomifensine had very slight releasing effects, whilst the reportedly selective dopamine uptake inhibitor, CDCI, produced only a slight effect at 5 μM. The neuroleptic agent, *cis*-flupenthixol (10 to 1000 nM) produced a dose-dependent decrease in the spontaneous release of [^{3}H]-ADTN (Figure 7), while the pharmacologically inactive *trans*-isomer did not influence the release (not shown). The ability of any of these agents to affect the K^+-evoked release of ADTN was not investigated.

Table 1 Inhibition of the uptake of [^{3}H]-ADTN into crude synaptosomes

Drug	IC_{50}(nM)
ADTN	140
Dopamine	168
Benztropine	180
Nomifensine	200
Noradrenaline	920
Amphetamine (i)	290
Amphetamine (ii)	3350
Imipramine	75000

Synaptosomes were incubated with [^{3}H]-ADTN at 37°C with the drug under investigation at concentrations of generally between 0.01 and 10 μM. Log dose inhibitor concentration was plotted against [^{3}H]-ADTN uptake as a percentage of control and, the IC_{50} values were read. Each point on the plots (not shown) was the mean of at least three determinations. The results for amphetamine are derived from (i) reserpine-treated and (ii) control, animals.

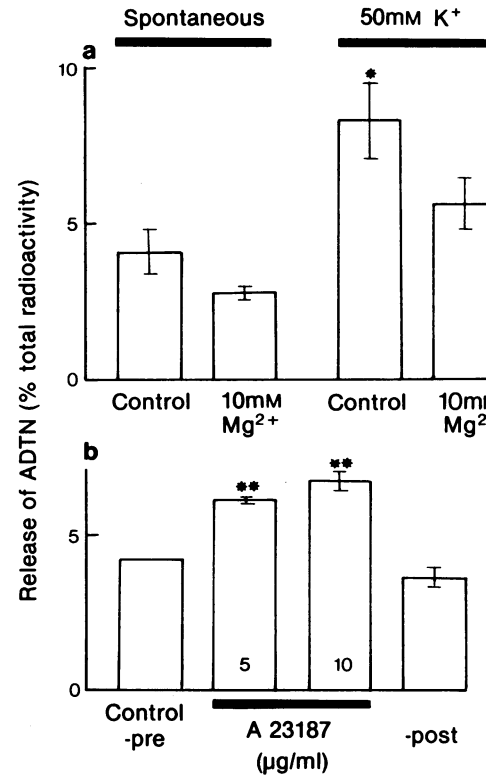

Figure 5 Calcium-dependency of the release of [^{3}H]-ADTN. Striatal slices were incubated for 20 min with [^{3}H]-ADTN and then transferred through a series of vials containing medium, as described in the text. The results are expressed as the fractional release, i.e. that percentage of the total radioactivity originally in the tissue, released per unit collection period. Control release is that release in the collection period immediately before the test period. (a) Effects of zero calcium and added 10 mM magnesium on the spontaneous and K^+-evoked release. Significance of difference of K^+-evoked release from control, $^*P < 0.05$ by *t* test. Effects of calcium depletion were not statistically significant. (b) Effect of the calcium ionophore, A23187; significance of difference from control, $^{**}P < 0.001$. Results are means of at least 4 observations in both (a) and (b); vertical lines show s.e. means.

Discussion

Although one previous study has reported a moderate ability of ADTN to inhibit the uptake of [^{3}H]-dopamine (IC_{50} = 850 nM) into striatal homogenates

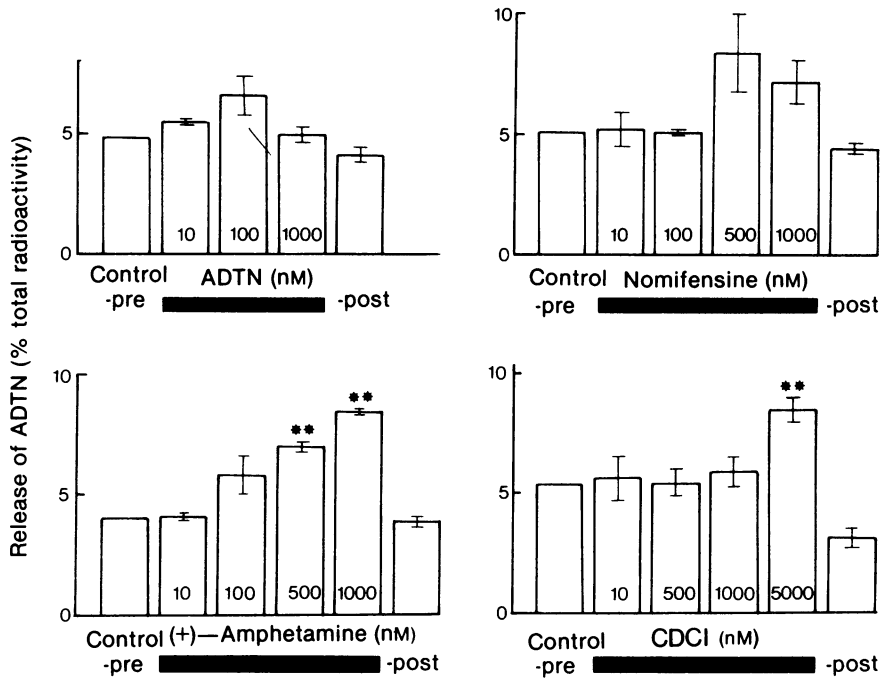

Figure 6 Effects of drugs on the release of [^{3}H]-ADTN. The experimental procedure was as described in the text and in the legend to Figure 5. Presence of the drug under test is indicated by the solid horizontal bars. Results are means of at least 4 observations. Vertical lines show s.e. means. Significance of difference of drug effects from control release, $^{**}P < 0.001$, by t test.

(Horn, 1974), until now, there has been no direct evidence in support of ADTN being a substrate for the dopamine carrier. We have demonstrated here that [^{3}H]-ADTN is avidly accumulated by both crude synaptosomes and striatal slices, the latter attaining a tissue: medium ratio of >170 after a 4 min incubation. Uptake was temperature-sensitive and readily depressed by a variety of metabolic inhibitors, indicating that in common with dopamine and a variety of other neurotransmitters, the transport mechanism is energy-dependent. Following uptake, ADTN was susceptible to metabolism, possibly by COMT. As has been reported for dopamine (Snyder & Coyle, 1969; Harris & Baldessarini, 1973; Holtz & Coyle, 1974; Del Rio & Madroñal, 1976; Hadfield, 1976; Davis, Davis & Halaris, 1977) the uptake of [^{3}H]-ADTN into striatal slices was also found to be mediated by a high-affinity, low capacity system; indeed, the two systems were indistinguishable. However, whilst Snyder & Coyle (1969) found only a single system in the striatum, with an apparent K_m of 0.4 μM, the Eadie-Hofstee plot for ADTN could be resolved into two separate components. This therefore suggests that ADTN may be a substrate for more than one carrier system in the striatum, although deviation from linearity in the plot need not necessarily implicate different transport systems. Results obtained from the cerebellum again indicated two components but these were of very much lower capacity and possibly represented transport into noradrenergic nerve terminals. In the peripheral nervous system for example,

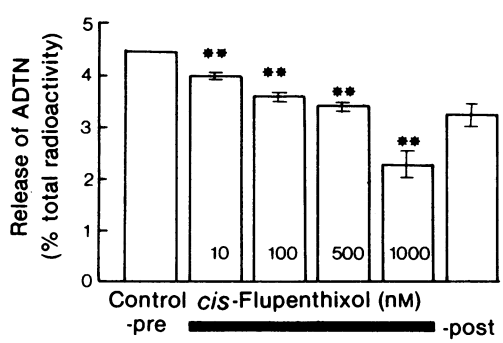

Figure 7 Inhibition of the spontaneous release of [^{3}H]-ADTN by *cis*-flupenthixol. Procedure as described in Figure 6. The presence of the neuroleptic is indicated by the solid bar. Each result is mean of at least 4 experiments. Vertical lines show s.e. means. Significance of difference from control release, $^{**}P < 0.001$.

[^{3}H]-ADTN can be accumulated by the noradrenergic terminals in the rat anococcygeus muscle (Doggrell & Woodruff, unpublished).

If indeed ADTN is a reasonably selective substrate for the high-affinity neuronal uptake mechanism for dopamine, then ADTN uptake should be influenced by each of those pharmacological agents with activity against dopamine uptake. Benztropine and nomifensine were found to be particularly potent, as indeed they are against dopamine itself (Hunt *et al.*, 1974) while imipramine, which has only a weak inhibitory action on dopamine uptake, was virtually inactive against the uptake of ADTN. The differential effects seen with amphetamine on dopamine uptake, depending upon whether tissue amine levels were depleted by reserpine (Horn, Coyle & Snyder, 1971) were found to be present for ADTN uptake, where the inhibitory potency of amphetamine was 12 times greater in a synaptosomal preparation from reserpine-treated rats. Very recently, CDCI has been reported to be a potent and specific competitive inhibitor of dopamine uptake in striatal systems (Herblin, 1977). The weak activity found in our experiments might be accounted for by the compound's very low solubility.

Thus, the pharmacological characteristics of ADTN uptake strongly suggest that it is being transported into dopaminergic nerve terminals. We do not know as yet whether ADTN can be accumulated like dopamine into 5-hydroxytryptaminergic nerve terminals but autoradiography with more highly labelled ADTN may help to clarify this possibility.

Investigation of the release of [^{3}H]-ADTN from striatal slices demonstrated a marked enhancement in the efflux when the medium K^+ concentration was elevated to 50 mM. Although it was not possible to show a clear-cut calcium-dependency in a calcium-free medium, probably because of the difficulty of depleting sufficiently the tissue calcium stores, the use of the calcium ionophore, A23187 resulted in an increased release of [^{3}H]-ADTN into the medium.

Although Coyle & Snyder (1969) originally reported that various anti-Parkinsonian drugs inhibited the uptake of labelled dopamine into striatal synaptosomes, recent reports have suggested that these pharmacological agents might be acting primarily as releasers of dopamine (Heikkila, Orlansky & Cohen, 1975; Baumann & Maitre, 1976). Raiteri and his co-workers have attempted to resolve this problem by the use of a synaptosome superfusion system, where re-uptake is eliminated (Raiteri, Cerrito, Cervoni, del Carmine, Ribera & Levi, 1977) and for example, have shown that both nomifensine and benztropine which have been suggested to have dopamine-releasing activity, were without effect at concentrations up to 10 μM and were considered pure uptake inhibitors. However, amphetamine was found to be a potent releaser at concentrations as low as 100 nM. In our experiments, amphetamine was a powerful releaser of [^{3}H]-ADTN at concentrations which were found not to influence uptake. Nomifensine was found to have an apparent weak releasing effect, but this was likely to be a reflection of the fact that reuptake had not been eliminated in our system. The result obtained with the neuroleptic, *cis*-flupenthixol was curious, since the inhibitory effects on ADTN release were clearly apparent at sub-micromolar (anaesthetic) concentrations. Additionally, the effect was specific since it was not shared by the therapeutically inactive *trans*-isomer. In relation to the release of [^{3}H]-dopamine, neuroleptics are known to elicit an opposite effect ie. an increase in the spontaneous release, but an inhibition of the electrically evoked release (Seeman & Lee, 1975). The significance of this effect is not clear. However, the cellular site from which the ADTN was being released is not known apart from its being of synaptosomal origin.

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