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CHANGES IN (^{3}H)-ADTN BINDING TO MICROSOMAL AND SYNAPTIC MEMBRANE FRACTIONS FROM RAT STRIATA FOLLOWING KAINIC ACID LESIONS

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Abstract—Specific binding of the dopamine agonist (^{3}H)-ADTN to rat striatal and microsomal membranes was examined. Both high (10 nM) and low (2 μM) affinity binding to each membrane fraction was found. Following kainic acid treatment the high affinity binding was significantly reduced, as was the basal level of adenylate cyclase, glutamic acid decarboxylase and choline acetyl transferase. Binding of the ligand to the low affinity site was, however, increased. These results are consistent with a postsynaptic localization of the high affinity (^{3}H)-ADTN binding site in rat striatal synaptic membranes.

The rigid dopamine analogue ADTN (2-amino-6, 7-dihydroxy-1, 2, 3, 4-tetrahydronaphthalene) first described by Woodruff [1] has potent dopaminergic activity in a number of systems [2]. It has recently been used as a ligand for the labelling of dopamine binding sites in a variety of preparations [3, 4]. An early report, using a preparation of ADTN with low specific activity, suggested the presence of a binding site with a dissociation constant in the μM range [5]. The presence of this site was later confirmed [4] and a binding site with a much higher affinity described. The rank order of potency of dopamine agonists in displacing (^{3}H)-ADTN binding and the close correlation with their ability to stimulate dopamine-sensitive adenylate cyclase suggested that this second high affinity site might represent the binding of (^{3}H)-ADTN to post-synaptic dopamine receptors.

This suggestion has been tested by examining (^{3}H)-ADTN binding and dopamine-stimulated adenylate cyclase activity in rats pretreated with the neurotoxin kainic acid. This compound causes selective degeneration of cell bodies in the striatum, leaving terminals and axons intact [6]. Such a procedure would be anticipated to reduce dopamine post-synaptic receptor activity.

MATERIALS AND METHODS

Membrane preparation. Crude synaptic membranes were prepared from rat striata as follows. A 10% (w/v) sucrose homogenate was centrifuged at 800 *g* for 10 min. The resulting supernatant was centrifuged at 17,000 *g* for 20 min. Synaptic membranes were prepared from the pellet (P_2) and microsomal membranes from the supernatant (S_2). The former was separated on a two-step discontinuous Ficoll–sucrose gradient (7.5 and 13% Ficoll in sucrose, w/v), at 43,000 *g* for 60 min in an SW27 rotor of a Beckman L5-65 ultracentrifuge. The synaptosomes separated as the middle layer on the gradient. They were removed by pipette, washed and lysed by homogenization (0.1 mm pestle clearance) in 50 mM Tris–Krebs' buffer, pH 7.4, containing 0.1% ascorbic acid and 10 μM nialamide. The microsomal membranes were obtained by centrifugation of the S_2 fraction at 100,000 *g* for 60 min. The pellet was washed, lysed and resuspended as above. All centrifugations were carried out at 4° and 0.32 M sucrose was made up routinely in 50 mM Tris–HCl, pH 6.5. Membranes prepared by this method were stable, if stored in liquid nitrogen, for periods up to one month.

(^{3}H)-*ADTN binding.* Binding assays were carried out in the presence and absence of 1 μM (±) ADTN and the specific binding (approximately 50 per cent of the total binding) obtained by subtraction. The incubation medium consisted of 100 μl membranes (200–300 μg protein) and 400 μl buffer. The reaction was started, after a 2 min preincubation, by the addition of (^{3}H)-ADTN (7.6 Ci/mmole) to a final concentration of 10 nM (high affinity site) or 2 μM (low affinity site). Incubation was for 10 min at 37° after which time the reaction was terminated by placing the tubes on ice. Free and bound ligand were separated by Millipore filtration, the bound fraction being washed with 10 ml ice-cold buffer. Binding is expressed as fmoles (^{3}H)-ADTN bound/mg protein.

Dopamine-sensitive adenylate cyclase. The dopamine-sensitive adenylate cyclase was assayed in rat striatal homogenates (25 vol., 2 mM Tris–HCl buffer, pH 7.0 containing 2 mM theophylline + 2 mM EGTA) by the method of Kebabian *et al.* [7]. Incubations in the presence and absence of drug were initiated by the addition of 0.5 mM ATP. Following 2.5 min at 37°, the reaction was terminated by placing the tubes in a boiling water bath for 2.5 min. Cyclic AMP content was assayed using a competitive binding protein assay [8] in aliquots of the supernatant. Results are expressed as pmoles cyclic AMP formed per mg protein.

Kainic acid lesions. Kainic acid (2 μg in 2 μl of 50 mM Tris-citrate buffer, pH 7.4) was stereotaxically injected at a rate of 0.2 μl/30 sec into the left caudate nucleus of male 200 g Wistar rats. The cannula was left in place for a further 1 min before

Table 1. Biochemical changes in striata from rats lesioned for 24 hr with kainic acid*

	N	Injected	Control	Change (%)
Glutamic acid decarboxylase (nmoles/hr/mg wet wt)	8	2.53 ± 0.10	3.80 ± 0.28	−33.4‡
Choline acetyltransferase (pmoles/hr/mg wet wt)	6	7.91 ± 1.11	15.79 ± 0.84	−49.9§
Striatal weight (mg wet wt)	8	27.5 ± 1.50	23.90 ± 1.10	+14.9†
Protein content (mg membrane protein/mg wet wt)	6	5.40 ± 0.69	6.37 ± 0.54	−15.2

* GAD and CAT levels were assayed as described in Materials and Methods, control values are from the contralateral striata. Lesions were produced by 2 μg kainic acid in 2 μl buffer injected over a 5 min period. Animals were killed 24 hr later.
† $P < 0.02$.
‡ $P < 0.01$.
§ $P < 0.001$.

withdrawing. The anaesthesia used was Nembutal (60 mg/kg i.p.) and the injection co-ordinates A + 7.9.L 2.6, V 4.8 according to the atlas of König and Klippel [9]. Animals were killed 24 hr later by cervical dislocation.

Enzyme assays. Glutamic acid decarboxylase was measured by the evolution of (^{14}C)-labelled CO_2 from DL-(1-^{14}C)-glutamic acid by the method of Roberts and Simonsen [10]. Cholineacetyl transferase was assayed by the method of Fonnum [11], utilizing liquid phase cation exchange. Protein estimations were by the method of Lowry *et al.* [12].

RESULTS

Verification of the kainic acid lesions. The kainic acid lesioned rats exhibited profound behavioural changes which were not apparent in the sham operated controls. These consisted of, in the first instance, bursts of 'corkscrewing' activity away from the lesioned side, followed by simple rotation in the same direction. These observations are similar to those described by Coyle and Schwarcz [13].

The lesion was accompanied by biochemical changes as shown in Table 1. The protein content was significantly reduced and the average striatal weight increased. The table also shows changes in glutamic acid decarboxylase (GAD) and choline acetyl transferase (CAT) markers of the GABA and acetyl choline containing interneurones of the striata, respectively. GAD levels were reduced by 33 per cent and CAT by 50 per cent in the lesioned striata compared with control levels taken from the contralateral striata. This is assumed to be a valid control since GAD activity in normal striata is 4.17 ± 0.45 [3] (nmoles/hr/mg wet wt) compared with 3.80 ± 0.28 [8] in contralateral to the injected striata and 3.80 ± 0.90 [3] in striata from sham-operated rats. These changes are similar to those reported by other workers [14] and demonstrate that valid lesions had been achieved. This was confirmed by a study of the morphological changes seen after kainic acid lesions where the lesioned striata exhibit a high degree of vacuolation compared with the structure of control tissue. Seven days after lesioning, the degenerative process had spread to the myelinated axons and with this in mind a time period of 24 hr was used to obtain maximum differentiation between loss of pre- and post-synaptic elements. Schwarcz and Coyle [15] showed that major degenerative changes had taken

Table 2. Effect of agonists and antagonists on high affinity binding to rat striatal and microsomal membranes*

	K_i (nM)	
Drug	Synaptic membranes	Microsomal membranes
Dopamine	210.0	N.T.†
Apomorphine	21.0	10.1
R(+)-ADTN	22.0	5.7
S(−)-ADTN	7500.0	417.0
(+) Butaclamol	12.8	1.1
(−) Butaclamol	5750.0	389.0

* High affinity binding to synaptic membranes was carried out using 10 nM (^{3}H)-ADTN and 1 μM R(+) ADTN as the displacing agent and to microsomal membranes with 20 nM (^{3}H)-ADTN using 1 μM (±) ADTN to define specific binding. The K_i values were obtained from log probit analysis, each drug being tested at not less than four concentrations, at least four times.
† N.T. = not tested.

Table 3. Changes in (^{3}H)-ADTN binding in kainic acid lesioned rat striatal membranes*

Binding fraction	N	Injected	Control	Change (%)
High affinity		fmoles/mg protein		
Synaptic membranes	7	41.6 ± 6.6	63.3 ± 7.5	−34.3†
Microsomal membranes	4	20.0 ± 4.6	80.4 ± 4.3	−75.1§
Low affinity		pmoles/mg protein		
Synaptic membranes	3	7.1 ± 0.6	5.7 ± 0.5	+21.9‡
Microsomal membranes	3	8.8 ± 0.8	5.4 ± 0.9	+61.3†

* Kainic acid (2 μg in 2 μl buffer) was infused into the left striatum for a 5-min period, animals were killed 24 hr later and the contralateral striata formed the control group. Binding was assayed in synaptic membranes with 10 nm (^{3}H)-ADTN and microsomal membranes with 20 nM (^{3}H)-ADTN. 1μM R(+)-ADTN was used to define specific binding in the synaptic membranes and 1μM (±)-ADTN in the microsomal membranes. Binding to the low affinity sites in both membranes preparations was with 2 μM (^{3}H)-ADTN and 1 mM dopamine as the displacing agent.

† $P < 0.02$.

‡ $P < 0.01$.

§ $P < 0.001$.

place within 48 hr and remained constant up to 21 days.

(^{3}H)-*ADTN binding*. Specific (^{3}H)-ADTN binding occurs not only to striatal membranes but also to microsomal membranes [16]. The effectiveness of dopamine agonists and antagonists in displacing the ligand from this site is shown in Table 2, where K_i values obtained from log probit analysis are compared with values obtained for synaptic membrane binding. It can be seen that the rank order of potency of dopaminergic agonists and antagonists is similar in both binding sites. Binding to both synaptic and microsomal membranes shows stereochemical specificity in that the agonist R(+)-ADTN is the active isomer in both and the antagonist (+)-butaclamol is much more active than the pharmacologically inactive (−)-butaclamol isomer. Thus the binding of (^{3}H)-ADTN in rats subjected to kainic acid lesions was compared in synaptic and microsomal membranes fractions with binding to the respective control (contralateral striata) membranes. In addition, the effect of kainic acid upon the high and low affinity binding sites was examined. The results of this study are shown in Table 3. It can be seen clearly that binding of the ligand at the high affinity site is significantly reduced in both synaptic and microsomal membranes, the reduction (75 per cent) of binding to the microsomal membranes being greater than that to the synaptic membranes (34 per cent). In contrast to this observation, ligand binding at the low affinity site was increased in both membrane fractions, again the increase being greater in the microsomal membrane fraction (61 per cent compared with 21 per cent).

Dopamine-sensitive adenylate cyclase. The dopamine-sensitive adenylate cyclase also shows changes following injection of kainic acid into rat striata. The changes are manifest by a loss of enzyme activity, as shown in Table 4, the EC_{50} values for dopamine remaining unchanged (3.0 μM in control striata and 2.7 μM in lesioned striata).

DISCUSSION

The agonist (^{3}H)-ADTN has been shown to bind to at least two sites in rat striatal synaptic membranes, a low affinity site [5] and a high affinity site [2]. Following kainic acid lesions, binding to the high affinity site was significantly reduced by an amount similar to the reduction in the dopamine-sensitive cyclase. Since there is much evidence to suggest that this enzyme is located entirely postsynaptically in the striatum [15, 17], this observation would suggest that the high affinity binding site is also associated with postsynaptic membranes. This observation suggests that (^{3}H)-ADTN is a suitable probe for mon-

Table 4. Effect of kainate lesions on striatal dopamine sensitive adenylate cyclase*

	N	Control	Kainate lesioned	Change (%)
Basal level (pmoles/mg/min)	20	203 ± 7	89 ± 6	−56†
+Dopamine (100μm) (pmoles/mg/min)	5	306 ± 8	135 ± 4	−56†
EC_{50} (μM)		3.0	2.7	

* Kainic acid (2 μg in 2 μl buffer) was infused over a 5 min period into the left striata, animals were killed 24 hr later, and the contralateral striata used as controls.

† $P < 0.001$.

itoring postsynaptic binding. This was suggested also by Quick *et al.* [18], who examined the potencies of dopamine, ADTN and (+)-butaclamol in displacing (^{3}H)-spiroperidol from frontal cortex and striatal membranes. Studies using the same radiolabelled ligand in 6-OH dopamine-treated rats, where dopamine receptor supersensitivity would be expected, and examining various dopaminergic ligands again showed that ADTN has a marked specificity for striatal membranes [19, 20]. Furthermore, evidence is accumulating to show that labelled dopamine antagonists such as spiroperidol and butaclamol bind to cortical striatal tract sites as well as to striatal sites [21].

In contrast to the high degree of specificity for agonists and antagonists and the marked stereospecificity described for the high affinity site, the low affinity binding site (2 μM) shows a rather high affinity for noradrenaline, low stereospecificity to the isomers of ADTN and a profound increase in activity following chronic haloperidol treatment [2]. These observations lead these authors to suggest that this binding site may represent binding of (^{3}H)-ADTN to an enzyme. The present study is in accord with this suggestion since following kainate lesions the binding was significantly increased, indicating a predominately presynaptic location. Enzyme activity has been shown to increase following kainate lesions, for example Coyle *et al.* [14] showed an increase in tyrosine hydroxylase in this situation.

This paper has also presented evidence for both high and low affinity binding sites in microsomal membranes. A high affinity binding site has previously been seen in this membrane fraction by Laduron *et al.* [22], using (^{3}H)-spiroperidol as the ligand, and by de Blas and Mahler [23], studying cholinergic receptor binding. In the present study the high affinity site found in microsomal membranes appears to have a similar rank order of potency to dopaminergic agonists and antagonists, to show stereospecificity, and to decrease its ability to bind the ligand following kainate lesions. Thus it appears to be similar to the high affinity binding site found in the purified striatal membranes. At least two explanations have been put forward to explain the presence of the microsomal site. De Blas and Mahler [23] suggested that it represented as yet unidentified synaptic elements which fractionate with the P3 pellet. A more likely explanation was proposed by Bergeron *et al.* [24], who showed enrichment of insulin receptor binding to the Golgi fraction of a P3 pellet and suggested that this might represent newly-synthesized receptors 'en route' to their final membrane location. When a neurone degenerates as, for example, after kainic acid treatment, protein synthesis would be expected to terminate, and the large loss of microsomal (^{3}H)-ADTN binding (75 per cent) might be a reflection of this. In any event, the finding of significant microsomal binding might be expected to alter the interpretation of studies using relatively crude membrane preparations.

In summary, the results presented are consistent with a postsynaptic localization of the high affinity (^{3}H)-ADTN binding site in rat striatal synaptic membranes.

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