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Evidence that (+)[^{3}H]amphetamine binds to acceptor sites which are not MAO-A

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The existence in rat hypothalamus of two binding sites for (+)amphetamine, with high [1–3] and low [2, 3] affinity has been observed. More precisely only those of lower affinity were discussed and characterized in regard of amphetamine pharmacological profile: a role in anorexia was postulated [2]. In a previous paper [3] we have noticed that several monoamine oxidase type-A (MAO-A) inhibitors and especially harmaline displace (+)[^{3}H]amphetamine ([^{3}H]AMPH) from its binding sites. Since [^{3}H]harmaline was used to label the MAO-A site [4] and that this compound exhibited a very good affinity for the high-affinity site [3] (which was very close to the one reported by Nelson *et al.* [4] for the [^{3}H]harmaline binding), it was suggested that MAO-A could represent the [^{3}H]AMPH high-affinity site [3, 5]. This study was conducted in order to verify this hypothesis.

We have also examined the possibility that (+)amphetamine could bind to a monoamine carrier since this drug inhibits the uptake of dopamine and noradrenaline *in vitro* [6]. Finally regional and subcellular properties of rat brain [^{3}H]AMPH-binding sites, in various incubation mediums, were studied.

Materials and methods

(+)[^{3}H]Amphetamine sulfate (sp. act. 15.1 or 18.6 Ci/mmole), was purchased from New England Nuclear, Boston, MA. (+)Amphetamine sulfate was from Coopération Pharmaceutique Française. Chloroquine and harmaline were obtained from Serva (Heidelberg, F.R.G.) and Sigma Chemical Co. (St. Louis, MO), respectively. Desipramine, nomifensine and clorgyline were synthesized by the Department of Organic Chemistry, Centre de Recherche Delalande. 1-Deprenyl was a gift from Prof. J. Knoll, Department of Pharmacology, Semmelweis University of Medicine, Budapest, Hungary. Other chemicals were of analytical grade and purchased from Merck (Darmstadt, F.R.G.).

Binding assay. Male Sprague–Dawley rats (150–200 g) were used. Brain regions were rapidly dissected on ice according to Glowinski and Iversen [7] and crude membranes were prepared as previously described [3]. Binding assay was conducted at 0°. Unless otherwise stated, 75 nM [^{3}H]AMPH was used. Specific binding was defined as the difference between the binding observed in the presence and in the absence of 10^{-2} M non radioactive (+)amphetamine sulfate. After 15 min incubation the ligand-receptor complex was separated by ultracentrifugation and the bound ligand was extracted according to Lesage *et al.* [3]. Incubation buffers were 50 mM Tris HCl, pH 7.4/500 mM NaCl/5 mM KCl or 50 mM Tris HCl, pH 7.4/5 mM EDTA or 10 mM Tris HCl, pH 7.4 supplemented with various sucrose concentrations. All assays were conducted generally in duplicate or triplicate (for pH and sucrose experiments). Unless otherwise stated, results are the mean of three independent determinations.

Subcellular fractionation. Intra- and extrasynaptosomal mitochondria and synaptic membranes were prepared from whole brain homogenates as described by Urwyler and Von Wartburg [8] using discontinuous density gradient of sucrose. Each fraction was resuspended in the Na^+/K^+ incubation medium (Ultraturrax, setting $\frac{3}{4}$ for 15 sec) and used immediately.

Data analysis. Data from competition experiments and saturation isotherms were analysed with a non-linear fitting program as essentially described by Munson and Rodbard [9]. In each case, models up to 3 binding sites were tested and the calculations were conducted on a 4052 Tektronix computer.

Protein concentrations were measured by the method of Lowry *et al.* [10] using bovine serum albumin as a standard.

Results and discussion

Specific [^{3}H]AMPH binding increased linearly with tissue concentration over the range of 0.1–1 mg of membrane protein. Assays were routinely conducted at 0.4 mg (final incubation volume 200 μl).

Results from [^{3}H]AMPH/harmaline displacement curves obtained with the three subcellular fractions are summarized in Table 1. Harmaline was chosen as displacer agent in regard of its high selectivity for MAO-A [4]. These experiments showed clearly that [^{3}H]AMPH-binding sites represent a complex mixture of sites since triphasic patterns were observed in intra- and extrasynaptosomal mitochondria (Fig. 1). Monoamine oxidase is a mitochondrial enzyme which the type-A, in contrast to the type-B form, is preferentially localized in nerve terminal mitochondria [8]. Thus MAO-A cannot be the acceptor site labelled by [^{3}H]AMPH since an enrichment of the high-affinity site in the intrasynaptosomal mitochondria fraction was not obtained. Moreover, the high-affinity site was still present in synaptic membranes. This was corroborated by the persistence of the amphetamine-binding sites after irreversible inhibition of MAO-A and -B due to a preincubation [11], in the presence of both clorgyline and 1-deprenyl in large excess, of the crude membrane preparation (data not shown).

The regional distribution of [^{3}H]AMPH-binding sites was also rather intriguing (Table 1): in the presence of a high- or a low-ionic strength medium the distribution of binding sites was almost completely reversed. The most striking differences were observed for cerebellum and striatum. Saturation isotherms were determined from rat hypothalamic membranes in two different incubation buffers. In each case a single class of binding site was apparent (Hill number close to unity) in the concentration range of [^{3}H]AMPH used (5–500 nM). The K_D values obtained in the presence (1.4 ± 0.4 μM) or absence (1.20 ± 0.12 μM) of Na^+/K^+ ions were very close, but the binding capacity (B_{max}) was greatly enhanced in the absence of ions (16.9 ± 0.4 vs 46.4 ± 3.4 pmoles/mg protein). It is impor-

Table 1. Regional distribution of [^{3}H]AMPH-binding in rat brain

Brain region	Tris + EDTA	Tris + Na^+/K^+
	(fmoles/mg of protein)	
Hypothalamus	618	102
Striatum	461	67
Olfactory bulbs	442	101
Hippocampus	423	86
Pons medulla	409	124
Thalamus	362	52
Cortex	349	74
Cerebellum	261	132

Results are the mean of 3 determinations which vary less than 5%. Buffer composition is described under Materials and Methods.

75 nM [^{3}H]AMPH was used and non-specific binding was defined in the presence of 10^{-2} M (+)amphetamine.

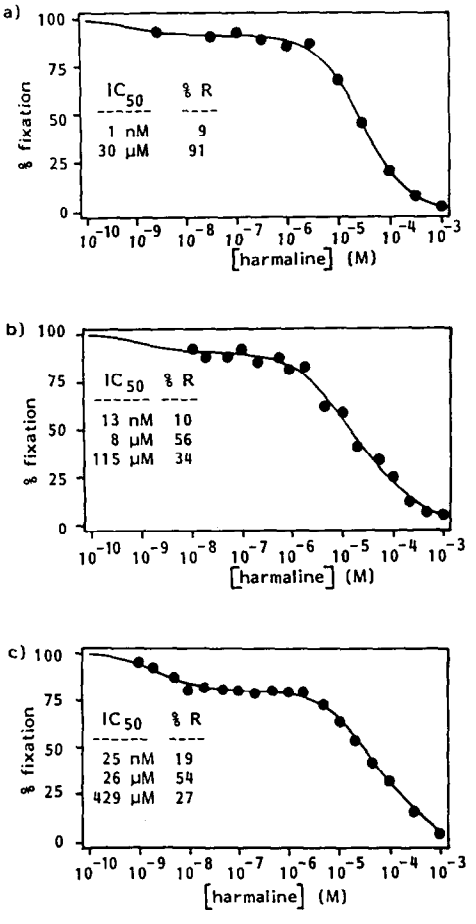

Fig. 1. Characterization of [^{3}H]AMPH-binding sites in subcellular fractions using harmaline as displacer in the presence of Na^+/K^+ ions. Displacement curves [IC_{50} and ratio (R) insert] are the mean of 2 separate experiments which vary less than 10%. (a) In synaptosomal membranes (data were best fitted with a two sites model; $P < 0.001$). (b) In intrasynaptosomal membranes (data were best fitted with a three sites model; $P < 0.001$). (c) In free mitochondria (data were best fitted with a three sites model; $P < 0.001$).

tant to notice that these B_{max} are rather compatible either with an enzyme, with a carrier capacity or more generally with an acceptor site.

Since (+)amphetamine inhibits both noradrenaline and dopamine uptake [6], the inhibition of [^{3}H]AMPH binding by nomifensine and desipramine was measured in hypothalamic crude membranes. Desipramine displaced [^{3}H]AMPH in a biphasic manner ($IC_{50} = 0.62 \pm 0.33$ and 55 ± 15 µM) but nomifensine was a poor inhibitor (15% inhibition at 30 µM). The inhibition of [^{3}H]AMPH binding by micromolar concentrations of desipramine can be correlated with recent results of Bönisch [12] who demonstrated that, besides a carrier-mediated uptake, a pronounced lipophilic entry of [^{3}H]AMPH exists in cultured PC-12 cells. This sodium-independent permeation was inhibited by desipramine at concentrations above 1 µM. Moreover, Hauger *et al.* [13] reported that [^{3}H]AMPH binding is inhibited by sodium ions or more generally by increased ionic strength: this is in good agreement with our present results. This prompted us to study the influence of osmotic pressure on [^{3}H]AMPH binding. In the presence of increased sucrose concentration (0.2–1 M) both specific and non-specific binding of [^{3}H]AMPH to rat hypothalamus membranes remain constant (data not shown). It can be concluded that amphetamine binding was not due to passive diffusion, a phenomenon observed in intact cells [12]. Moreover, chloroquine was a poor inhibitor of [^{3}H]AMPH binding to hypothalamic membranes in the presence ($IC_{50} = 200 \pm 30$ µM) or absence ($IC_{50} = 80 \pm 11$ µM) of Na^+/K^+ ions. Since chloroquine is a lysosomotropic drug, known to inhibit lysosomal uptake [14], this confirmed that [^{3}H]AMPH binding was not the result of a trapping process. Finally we studied the influence of pH on [^{3}H]AMPH binding in the presence of Na^+/K^+ ions using rat hypothalamic membranes. Specific binding was increased nearly 3-fold between pH 7 and 8 (Fig. 2). Non-specific binding remained constant for the same pH range. It is important to notice that under these pH conditions, the ratio of "free base/protonized amine" range between 0.1–0.3% (pKa AMPH, 9.9 at 20°, see [15]).

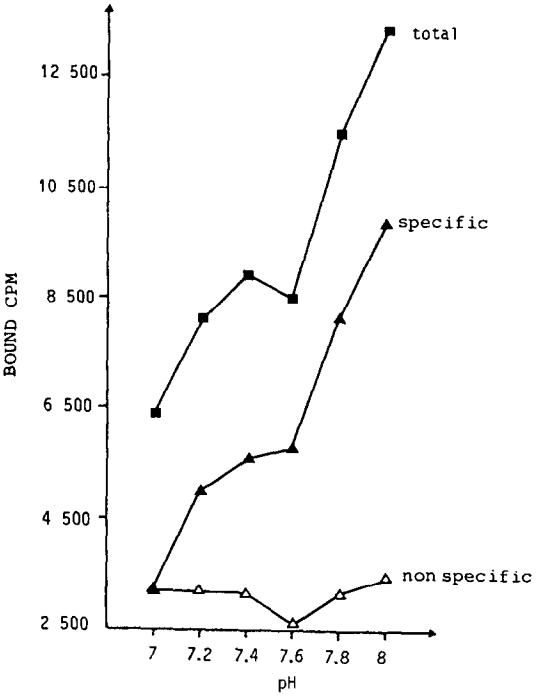

Fig. 2. Influence of pH on [^{3}H]AMPH-binding to rat hypothalamic membranes in the presence of Na^+/K^+ ions.

These results taken together confirm the heterogeneity of [^{3}H]AMPH-binding sites and strongly support the hypothesis that the drug could bind to membrane-acceptor sites which are neither a receptor nor MAO-A. The effects of varying pH or salt concentrations suggest that both ionic and hydrophobic interactions are present. Our previous study [3] concerning (±)*p*-chloroamphetamine, which exhibited a greater affinity for [^{3}H]AMPH sites than did (+)amphetamine, supports the existence of hydrophobic interactions. In addition the acceptor sites for [^{3}H]AMPH can be membrane phospholipids since amphiphilic drugs possessing structural analogies with amphetamine (e.g. chlorphentermine and phentermine) exhibit binding characteristics to phospholipids [16] which are similar to those of [^{3}H]AMPH binding.

In summary, the present data demonstrate that [^{3}H] AMPH binding does not reflect an accumulation process but rather a binding phenomenon to an acceptor site which is probably a polar lipid but not MAO-A. Our results and the multiple biochemical actions of amphetamine (e.g. inhihition of dopamine and noradrenaline uptake [6] and inhibition of MAO-A [17]) lead to the conclusion that [^{3}H]AMPH is not suitable for biochemical *in vitro* study.

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The carcinogen, 7-hydroxymethyl-12-methylbenz[*a*]anthracene, is activated and covalently binds to DNA via a sulphate ester

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7-Hydroxymethyl-12-methylbenz[*a*]anthracene(7-HMBA), a potently carcinogenic major metabolite of 7,12-dimethylbenz[*a*]-anthracene (DMBA) in rat liver [1–3], has recently been demonstrated to induce His$^+$ reverse mutation in *Salmonella typhimurium* TA 98 to a remarkable extent in the presence of liver cytosol fortified with 3′-phosphoadenosine 5′-phosphosulphate (PAPS) and to a slight extent in the presence of liver microsomes or a 9000 *g* supernatant fraction fortified with NADPH [4]. From the cytosol-PAPS system, a highly reactive 7-hydroxymethyl sulphate ester of 7-HMBA has been isolated and identified as a directly acting mutagenic metabolite [4]. The sulphate ester conjugate (7-HMBA sulphate) is yielded at a significant rate by cytosolic sulphotransferase and inactivated by cytosolic glutathione *S*-transferase in the presence of glutathione (GSH) to form an unreactive and non-mutagenic GSH *S*-conjugate [5]. In addition, biologically formed 7-HMBA sulphate bound covalently through its 7-methylene carbon with loss of a sulphate anion to nucleophilic residues of amino acids consisting in hepatic cytosolic proteins; *S*-cysteine, ε-*N*-lysine, and *S*-methionine adducts of the carcinogen have been isolated from the digested proteins and identified with the corresponding synthetic specimens [6].

These facts might provide us an important clue to solve the long-arising question about the significant increase in carcinogenicity of the weak carcinogen, benz[*a*]anthracene (BA), up to the highest level among polynuclear aromatic hydrocarbons by methylation at the "L-region" (7- and 12-positions) of BA as seen in DMBA, 7-methyl-BA and 12-methyl-BA [7].

Sulphate esters have long been putative, biologically reactive intermediates since Miller and his co-workers proposed them for the metabolic activation of the proximate carcinogens such as *N*-OH-FAA, *N*-OH-MAB and 1′-hydroxysafrole [8–12]. However, they have not yet succeeded in detecting the sulphates from biological systems. *N*-Hydroxyl derivatives of carcinogenic aromatic amines have also been demonstrated to be activated at weakly acidic pH, probably possible in urinary bladder [13], through their *O*-acetates [14], *O*- [15, 16] and *N*-glucuronides [13], aminoacyl esters [17] or nitrosyl radicals