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In-vivo (+)-[^{3}H]Fenfluramine Binding to Rat Brain: Biochemical and Autoradiographic Studies

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Abstract—The in-vivo binding of (+)-[^{3}H]fenfluramine to rat brain regions is saturable, as shown by the inhibition curves obtained by co-injecting increasing concentrations of unlabelled (+)-fenfluramine: at 2·5 mg kg^{-1} the inhibition of total bound radioactivity was maximal in all regions. The regional distribution of (+)-[^{3}H]fenfluramine specific binding sites (hypothalamus > striatum = cortex > brainstem > hippocampus > cerebellum) closely parallels the regional distribution of 5-hydroxytryptamine uptake. Computer-assisted quantitative autoradiography confirms these findings. The IC50 of (+)-fenfluramine for inhibition of its binding in-vivo is below 0·25 mg kg^{-1}, compatible with the presence of high affinity sites. While the physiological role of (+)-[^{3}H]fenfluramine binding sites in the brain, particularly in hypothalamic nuclei, is being investigated, it has been found that in-vivo labelling could also be obtained in the periphery, in lung and renal cortex. The possibility that this peripheral binding is due to the presence of blood platelets cannot be ruled out.

Stereospecific, saturable, high-affinity (+)-[^{3}H]fenfluramine binding to rat brain membranes in-vitro has been recently described (Mennini et al 1988). The binding is inhibited by 5-hydroxytryptamine (5-HT) uptake inhibitors, but, unlike [^{3}H]imipramine binding, it is inhibited by NaCl (Garattini et al 1987). Other drugs such as anorectics, belonging to different chemical classes, inhibit (+)-[^{3}H]fenfluramine binding with different potencies, not correlated to their efficacy in reducing food intake (Garattini et al 1987). Thus the role of these binding sites in the brain is not clear.

We now describe the regional distribution of in-vivo (+)-[^{3}H]fenfluramine binding, and its in-vivo saturability, demonstrated by the inhibition of co-injected unlabelled (+)-fenfluramine.

Materials and Methods

Male rats (about 200 g) were injected intravenously with 2·8 nmol of (+)-[^{3}H]fenfluramine (45 μCi/rat) dissolved in 0·5 mL of 0·9% NaCl in the absence or presence of different concentrations of unlabelled (+)-fenfluramine (final doses from 0·25 to 10 mg kg^{-1}). Two minutes later, the rats were decapitated and brain areas (hippocampus, striatum, hypothalamus, cortex, cerebellum and brainstem) and peripheral tissues (lung, kidney, heart, diaphragm, liver) were rapidly dissected and homogenized in about 30 vol of ice-cold Na-K phosphate buffer, 50 mM pH 7·4, using an Ultra-Turrax TP 1810 (20 s). Two samples of 0·5 mL were put directly into two scintillation vials, containing 10 mL of scintillation cocktail (Filter Coult, Packard), and counted for radioactivity in Beckman LS-7500, liquid scintillation-spectrometer with a counting efficiency 45%. These samples represent the "total radioactivity" measurement.

Two 0·5 mL samples were filtered through Whatman GF/B filters while another two samples were filtered after 30 min incubation at 37°C in the presence of 10 μM unlabelled (+)-fenfluramine to induce in-vitro dissociation of the ligand. The filters were then counted for radioactivity in 10 mL of Filter Count, as above. These samples represent the "total bound radioactivity" and the "in-vitro non-specific bound radioactivity", respectively. Free radioactivity was calculated as the difference between total and bound radioactivity. Protein content in the homogenates was determined by Peterson's method (1977) using bovine serum albumin as standard. For autoradiographic studies whole brains from animals injected with (+)-[^{3}H]fenfluramine were frozen and sliced (14 μm thick) using a cryostat, according to the Paxinos & Watson (1982) rat brain atlas. The slices were then thaw-mounted on gelatin-coated slides and exposed, together with brain paste [^{3}H] standards, on tritium sensitive film (Hyperfilm, Amersham) for three months at −50°C. Computer-assisted densitometric analysis was done using the RAS 1000 image analyser (Amersham).

(+)-[^{3}H]Fenfluramine (spec. act. 16 Ci mmol^{-1}) was obtained from CEA, France; unlabelled (+)-fenfluramine from Servier Laboratories, Neully, France. Other chemicals were from commercial sources.

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Results

Biochemical assay

Two minutes after i.v. injection of 45 μCi (about 3 nmol) of (+)-[^{3}H]fenfluramine the total radioactivity was measured in different rat brain regions (Table 1, 1st column). The value found in the cortex (443 fmol (mg prot)$^{-1}$) was significantly higher ($P < 0·01$) than those found in the other brain regions. None of the values were significantly influenced by co-injection with unlabelled (+)-fenfluramine (up to 10 mg kg^{-1}), as shown in Table 1, 4th column. Total bound radioactivity, measured by filtering an aliquot of brain homogenate, was always lower than 40% of total radioactivity (Table 1, 2nd column) and was significantly reduced by co-injection of different doses of unlabelled (+)-fenfluramine (from 0·25 to 10·0 mg kg^{-1}) (Fig. 1). The inhibition obtained with 2·5 mg kg^{-1} was clearly maximal, and no further inhibition could be obtained with 5, 7·5 or 10 mg kg^{-1} (data not shown).

Table 1. In-vivo (+)-[^{3}H]fenfluramine binding in different areas of rat brain and some peripheral tissues. Male rats were injected i.v. with 45 μCi (3 nmol) of (+)-[^{3}H]fenfluramine in absence (control) or presence of unlabelled (+)-fenfluramine (2·5 or 5 mg kg^{-1} for central and peripheral regions, respectively). Two minutes later the rats were killed and the dissected brain areas were homogenized. Two aliquots of the suspension were directly counted for radioactivity (total radioactivity), two aliquots were filtered through GF/B filters (total bound radioactivity) and two aliquots were incubated for 30 min at 37°C in presence of 10 μM unlabelled (+)-fenfluramine and filtered as above (in-vitro, non-specific bound radioactivity). The values, expressed as fmol (mg protein)$^{-1}$, are given as mean ± s.d. of four rats.

	Control			(+)-F Co-injection		
Area	Total radioactivity	Total bound radioactivity	In-vitro non-spec. bound radioactivity	Total radioactivity	Total bound radioactivity	In-vitro non-spec. bound radioactivity
Hypothalamus	222±34	87±9	30±12	328±37	30±37	21±5
Striatum	302±35	76±10	17±2	406±40	38±1	22±3
Brainstem	256±33	84±10	14±1	348±12	56±5	22±5
Hippocampus	232±41	63±10	14±3	282±11	45±5	13±8
Cortex	443±58	134±17	28±5	584±22	98±23	33±8
Cerebellum	300±37	104±12	31±5	385±12	92±20	41±6
Lung	1355±211	382±55	62±5	579±31	83±14	23±9
Heart	141±46	28±10	5±3	96±2	14±1	3±1
Diaphragm	116±37	15±6	3±1	89±2	4±1	2±1
Kidney	203±45	75±20	13±6	172±26	36±6	11±3
Liver	28±10	18±7	2±1	24+1	12±1	2±1

In-vivo incubation of the homogenate with 10 μM cold (+)-fenfluramine determined a dissociation of the in-vivo bound ligand. The radioactivity remaining in the tissue can be considered as non-specific bound radioactivity. However. these values (Table 1, 3rd column) are significantly lower than those determined by co-injection with a saturating concentration of unlabelled (+)-fenfluramine (2·5 mg kg^{-1}, Table 1, 5th column). Moreover, the radioactivity bound in-vivo after 2·5 mg kg^{-1} (+)-fenfluramine can be further decreased by in-vitro dissociation (Table 1, 6th column).

The non-specific binding determined in-vitro accounted for 5–10% of total radioactivity and was similar in all the brain regions considered. Therefore using these values to calculate the specific (+)-[^{3}H]fenfluramine binding, a method common for in-vivo binding, we found that the relative regional distribution of the specific binding values closely parallel to that of total bound, with the cortex showing levels significantly ($P < 0·05$) higher than the other brain areas.

From the data presented in Table 1, the in-vivo (+)-[^{3}H]fenfluramine specifically bound could also be calculated, assuming the non-specifically bound as that obtained by co-injecting 2·5 mg kg^{-1} of unlabelled drug. The regional distribution of (+)-[^{3}H]fenfluramine specific binding calculated in this way indicated highest concentrations in hypothalamus (57 fmol (mg protein)$^{-1}$), followed by striatum (38), cortex (36), brainstem (28), hippocampus (18) and cerebellum (12). From the data presented in Fig. 1 it was also

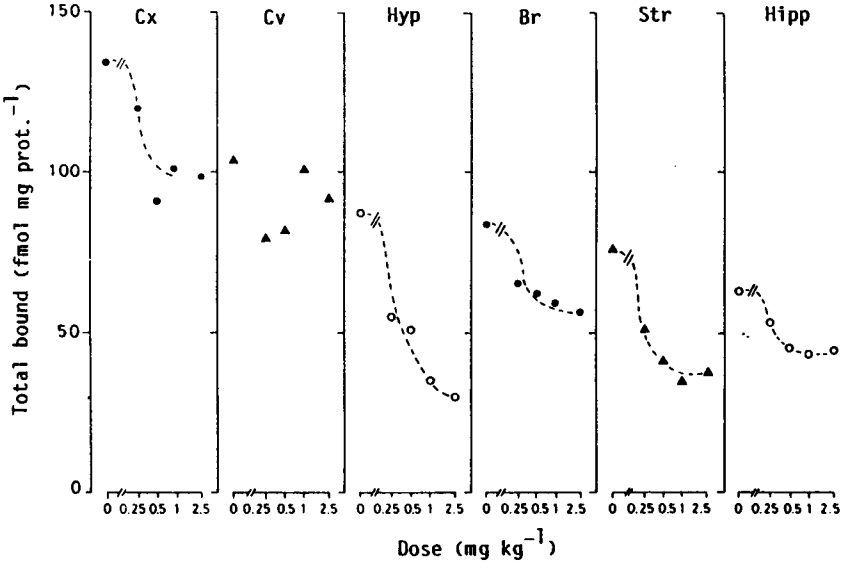

FIG. 1. In-vivo inhibition of (+)-[^{3}H]fenfluramine binding by unlabelled (+)-fenfluramine. Male rats, about 200 g, were injected intravenously with 3 nmol of (+)-[^{3}H]fenfluramine (45 μCi/rat) dissolved in 0·5 mL of 0·9% NaCl in the absence or presence of different concentrations of unlabelled (+)-fenfluramine (final doses from 0·25 to 10 mg kg^{-1}) and killed 2 min later. Membrane bound total radioactivity, measured as described in Materials and Methods, is plotted against doses of co-injected unlabelled (+)-fenfluramine (log. scale). Each point represents the mean value of 4 animals per group, with standard deviation lower than 15%. Cx: cortex; Cv: cerebellum; Hyp: hypothalamus; Br: brainstem; Str: striatum; Hipp: hippocampus.

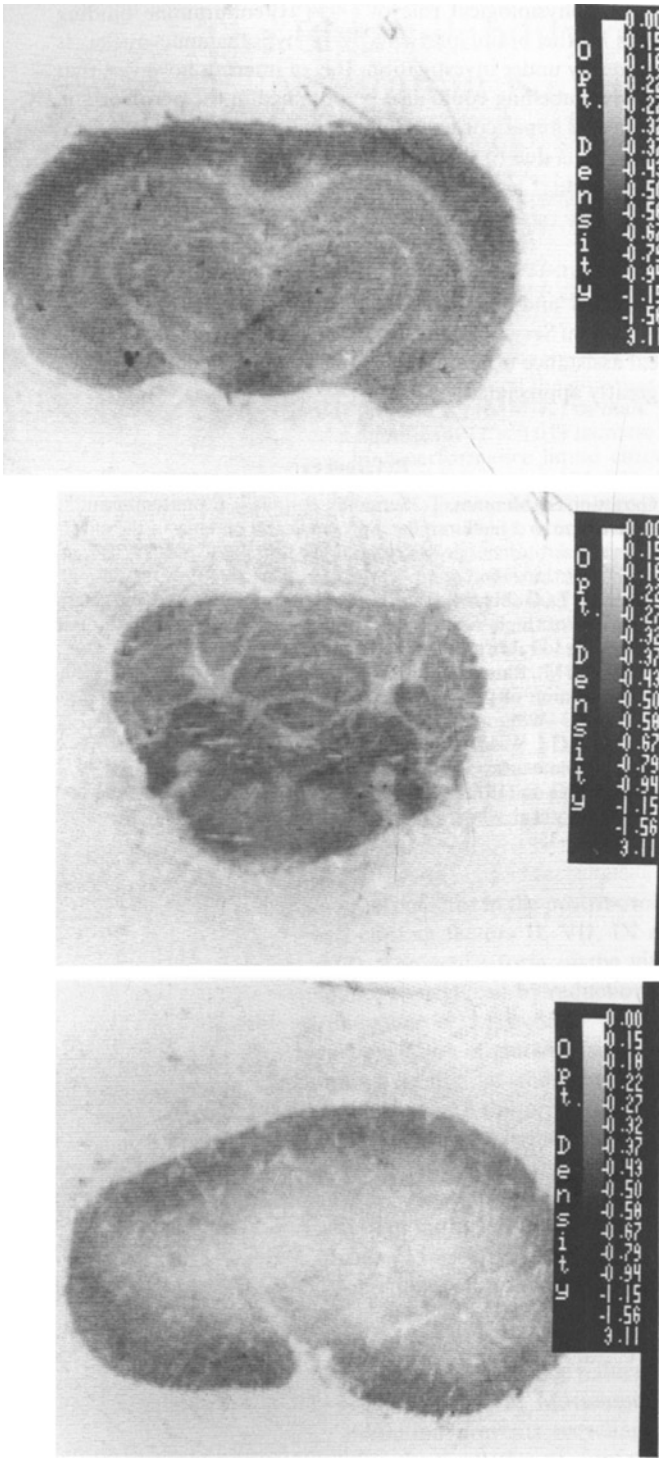

FIG. 2. In-vivo localization of (+)-[^{3}H]fenfluramine binding. Representative autoradiograms from brain (top), cerebellum (middle) and kidney (bottom) are shown. Dark regions represent areas with high levels of total radioactivity.

possible to calculate the IC50 values for in-vivo inhibition of (+)-[^{3}H]fenfluramine binding. The lowest were for hypothalamus and striatum (0·15 ± 0·05 and 0·13 ± 0·01 mg kg^{-1}, respectively); intermediate in hippocampus (0·23 ± 0·07) and highest in cortex and brainstem (0·31 ± 0·06 and 0·32 ± 0·11 mg kg^{-1}, respectively). The IC50 value in the cerebellum could not be established.

Table 2. In-vivo (+)-[^{3}H]fenfluramine binding in different areas of rat brain and kidney: autoradiographic studies. Male rats were injected i.v. with 45 μCi (3 nmol) of (+)-[^{3}H]fenfluramine in 0·5 mL saline and killed 2 min later. The brains were removed and immediately frozen on dry ice. 14 μm sections were then cut and used for autoradiography as described in materials and methods. Data are means of determinations in different plates from two rat brains. In parentheses are reported the percentage of variation of such determinations. Structures are referred as in Paxinos & Watson (1982) rat brain atlas.

Area	Structures	Total radioactivity (fmol (mg tissue)$^{-1}$)
Cortex	Cg	43 (16%)
	FrPaM	44 (11%)
	FrPaSS	41 (22%)
Cerebellum		41 (15%)
Striatum	CPu	28 (39%)
Hypothalamus	LH	25 (8%)
	VMH	27 (22%)
Hippocampus	CA1	29 (41%)
	CA2	18 (44%)
	CA3	23 (30%)
	CA4	25 (28%)
	DG	18 (17%)
White Matter		13 (38%)
Kidney	Cortex	47 (17%)
	Medulla	13 (31%)

(+)-[^{3}H]fenfluramine binding was also found in peripheral tissues (Table 1). Total radioactivity in the lung was significantly higher ($P < 0·01$) than in the other organs. Free radioactivity was higher than 60% of total radioactivity, except in the liver where it represented only 33% of total radioactivity. The radioactivity bound was significantly reduced by in-vitro dissociation with an excess of unlabelled (+)-fenfluramine and by co-injection with 5 mg kg^{-1} (+)-fenfluramine in all tissues except the liver. Specific (+)-[^{3}H]fenfluramine binding was calculated as that displaceable by in-vivo co-injection with unlabelled drug: highest levels were found in the lung (Table 1).

Autoradiography

In-vivo distribution of (+)-[^{3}H]fenfluramine was detected by autoradiographic analysis of slices from brain and kidney (Fig. 2). The distribution of total radioactivity in the brain, both qualitatively and quantitatively, confirmed that obtained in biochemical assays (Table 2). This approach also permitted measurement of the radioactivity present in smaller portions of each area, i.e. lateral and ventromedial nuclei of hypothalamus where similar distribution was found. In the kidney there was a clear difference between cortex and medulla (47 versus 13 pmol (g tissue)$^{-1}$).

Discussion

The present paper reports on in-vivo labelling of (+)-[^{3}H]fenfluramine binding sites in the brain of rats killed 2 min after intravenous injection of the tracer: at this time less than 5% of (+)-[^{3}H]fenfluramine is converted to (+)-norfenfluramine (Caccia, personal communication). In-vivo binding of (+)-[^{3}H]fenfluramine was saturable, as shown by the inhibition curves obtained by co-injecting increasing concentrations of unlabelled (+)-fenfluramine; with 2·5 mg kg^{-1}, inhibition of total bound radioactivity was maximal in all

regions (Fig. 1), enabling us to use these samples to calculate the specific binding.

Plasma concentrations of (+)-fenfluramine 5 min after i.v. injection of 2·5 mg kg^{-1} are about 3 nmol mL^{-1} (Caccia, personal communication); in this condition brain concentrations should be largely above the affinity of (+)-[^{3}H]fenfluramine for its receptors, determined in-vitro (Garattini et al 1987; Mennini et al 1988) and thus compatible with the maximal displacement obtained.

The regional distribution of (+)-[^{3}H]fenfluramine specific binding sites (hypothalamus > striatum = cortex > brainstem > hippocampus > cerebellum) closely parallels the regional distribution of 5-HT concentrations or 5-HT uptake (Palkovits et al 1981), suggesting that (+)-[^{3}H]fenfluramine binding sites are associated with 5-HT neurons.

On incubating the homogenate in the presence of an excess of unlabelled (+)-fenfluramine (10 μM), we obtained significantly lower "non-specific" binding values. Consequently, the calculated "specific" binding was higher and the relative regional distribution was different too (paralleling the distribution of total radioactivity in the brain). The cerebellum is the most impressive case: when specific (+)-[^{3}H]fenfluramine binding was calculated using the value obtained in-vitro, this was one of the brain regions with the highest density of binding sites (Garattini et al 1987). However, when we used the value obtained in-vivo it showed practically no specific binding.

These data underline the importance of assessing non-specific binding in-vivo, particularly using a ligand with a high free proportion. The IC50 of (+)-fenfluramine for inhibition of its binding in-vivo is below 0·25 mg kg^{-1}, compatible with the presence of high-affinity sites.

The physiological role of (+)-[^{3}H]fenfluramine binding sites in the brain, particularly in hypothalamic nuclei, is currently under investigation. It is of interest, however, that in-vivo labelling could also be obtained in the periphery, in lung and renal cortex. The possibility that this peripheral binding is due to the presence of blood platelets cannot be ruled out.

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