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In vitro and in vivo Disposition of ^{3}H-Methiothepin in Brain Tissues

Relationship to the Effects of Acute Treatment with Methiothepin on Central Serotoninergic Receptors

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Summary. A single treatment with a large dose of methiothepin (20 mg/kg, i.p.) induced, as early as the 2nd day after injection, a significant increase (+20–35%) in the number of specific binding sites for ^{3}H-5-HT in forebrain areas, particularly the hippocampus. Experiments with ^{3}H-methiothepin indicated that the drug remained firmly bound to brain membranes thus maintaining a local concentration high enough to effectively block 5-HT receptors for 10–12 h after its peripheral administration. Accordingly, it can be concluded that the occupancy of central 5-HT receptor sites by methiothepin for several hours was sufficient to induce a supersensitivity phenomenon within the two following days.

Although ^{3}H-methiothepin was a useful marker for analyzing the disposition and the kinetics of the 5-HT antagonist in brain tissues, it could not be used as a specific ligand of 5-HT receptors in brain since under in vitro as well as in vivo conditions most of ^{3}H-methiothepin bound to non-specific sites, especially to the lipid component of the membranes.

Key words: Receptors – ^{3}H-5-HT – ^{3}H-spiroperidol – ^{3}H-methiothepin binding – 5-HT-sensitive adenylate cyclase – Supersensitivity.

Introduction

Bennett and Snyder (1976) postulated that serotonin (5-HT) receptors exist as two forms, one exhibiting higher affinities for agonists, the other having better affinities for antagonists. Using ^{3}H-5-HT as a ligand, several authors have studied the characteristics of the "agonist" form in the brain of various species (Bennett and Snyder, 1976; Fillion et al., 1978; Nelson et al., 1978; Steigrad et al., 1978; Whitaker and Seeman, 1978a) including man (Shih and Young, 1978). Studies on the "antagonist" form are far less developed mainly because until recently only a mixed agonist-antagonist, ^{3}H-d-LSD, has been available as an appropriate ligand (Bennett and Snyder, 1976; Fillion et al., 1978; Whitaker and Seeman, 1978b). In fact, in order to first prove the existence of this "antagonist" form and then to further examine its properties, the best ligand would be a pure antagonist with a high specific radioactivity.

Very recently, several studies with ^{3}H-spiroperidol have indicated that, under certain conditions, this blocker might be a specific ligand of the "antagonist" form of 5-HT receptors (Creese and Snyder, 1978; Howard et al., 1978; Leysen and Laduron, 1977; Leysen et al., 1978; Quick et al., 1978). However, ^{3}H-methiothepin could be a better ligand since at present this drug is the only one which exerts all of the expected properties of a central 5-HT blocker. Indeed, in vitro, methiothepin inhibits both the high affinity binding of ^{3}H-5-HT to synaptosomal membranes (Bennett and Snyder, 1976; Nelson et al., 1978) and the stimulatory effect of 5-HT on brain adenylate cyclase (Bourgoin et al., 1977; Enjalbert et al., 1978). Furthermore, in vivo, this drug accelerates the turnover of 5-HT presumably by triggering a positive feed-back process linked to the blockade of 5-HT receptors (see Bourgoin et al., 1977; Monachon et al., 1972). Finally, in agreement with these biochemical data, electrophysiological studies in the cat indicated that methiothepin is the most potent blocker of central 5-HT receptors currently available (Ruch-Monachon et al., 1976). Therefore, a new procedure was devised for the synthesis of tritiated methiothepin of high specific activity. Attempts were then

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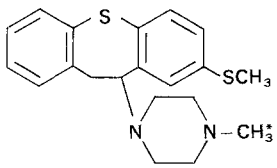
made to use this radioactive molecule as a ligand of 5-HT receptors both in vitro and in vivo. Unfortunately, the non-specific binding of ^{3}H-methiothepin was too high to characterize the "antagonist" form of 5-HT receptors in membranes from the rat brain. However, the kinetic analysis of the dispositon of ^{3}H-methiothepin in brain suggested that this drug remained firmly bound to membranes for at least 10–12 h after its peripheral administration. This long term occupancy of the 5-HT receptors might explain the subsequent increase in the number of ^{3}H-5-HT high affinity binding sites which occurred in the hippocampus as early as the second day after an acute peripheral injection of methiothepin.

Materials and Methods

Chemicals

Labelled Compounds. (α-^{32}P)ATP (sodium salt, 10–20 Ci/mmole, New England Nuclear), (G-^{3}H)-5-hydroxytryptamine creatinine sulphate (^{3}H-5-HT, 12–15 Ci/mmole, Radiochemical Centre, Amersham), S-adenosyl-L-(methyl-^{3}H)methionine (5 Ci/mmole, Radiochemical Centre, Amersham), (phenyl-4-(n)-^{3}H)spiperone (^{3}H-spiroperidol, 20 Ci/mmole, Radiochemical Centre, Amersham), (^{3}H-G)-haloperidol (7.86 Ci/mmole, New England Nuclear).

Synthesis of N-(methyl-^{3}H)-methiothepin



8-(methylthio)-10 piperazino-10,11 dihydrodibenzo (b, f) thiepin (N-desmethyl-methiothepin) 0.09 mmol as free base in 1 ml of methanol was N-methylated by catalytic reductive alkylation with formaldehyde (0.1 ml of a 35% solution) and tritium in the presence of palladium [15 mg of prereduced 5% Pd/C catalyst adsorbed 1.6 ml (NTP) tritium gas]. After the usual work-up, (N-methyl-^{3}H)methiothepin was purified by preparative thin layer chromatography (TLC) on Silicagel G (solvent S_1: ethanol: 9; water: 1 by vol. $R_f = 0.26$). The radiochemical purity was checked by TLC on Silicagel G using two different solvents: S_1 and S_2 (pyridine: 6; acetone: 3; dichloromethan: 1; water: 1, by vol. $R_f = 0.60$) and found to be of 97–98%. No significant degradation was noted when ^{3}H-methiothepin was kept in ethanol at −30° C for at least 8 months.

The U.V. spectrum coincided with the U.V. curve of cold methiothepin. Specific activity was 4.5 Ci/mmole. The tritium NMR spectrum gave one single signal at δ 1.72 p.p.m.

Sodium borotritide reduction in acetic acid of 8-(methylthio)-10-(4-methyl-piperazino) dibenzo (b,f) thiepin (Kaplan and Kyburz, 1973; Sindelar et al., 1975) as well as catalytic reduction with tritium were attempted and found to be unsatisfactory.

Other Compounds. Pargyline (Abbott), (+)-butaclamol (Ayerst), serotonin creatinine sulphate (Merck), methiothepin (Hoffmann-LaRoche), *d*-LSD (Sandoz), promethazine (Specia), chlorpromazine (Rhône-Poulenc), haloperidol (Rhône-Poulenc), metergoline (Farmitalia), dopamine (Calbiochem), dithiothreitol (Calbiochem), β-mercaptoethanol (Sigma), Ficoll 400 (Pharmacia). All other compounds were the purest commercially available (Merck, Prolabo).

Animals

Male Sprague-Dawley rats were housed in a controlled environment (24° C, 60% relative humidity, alternate cycles of 12 h light and 12 h darkness, food and water ad libitum) for at least 7 days before use. The animals (250–350 g) were killed by decapitation and all subsequent steps were carried out in the cold (0–4° C).

Methods

Partition of Labelled Ligands Between Octanol and Aqueous Layers

^{3}H-methiothepin, ^{3}H-spiroperidol, ^{3}H-haloperidol or ^{3}H-5-HT (35 nCi in 10 µl of water) was added to disposable plastic tubes containing 1 ml of octanol and 1 ml of buffer between pH 6.0 and 8.5 (0.05 M Na/K_2 phosphate buffer pH 6.0, 6.5, 7.0 and 7.5 or 0.05 M Tris-HCl buffer pH 7.5, 8.0 and 8.5). The mixture was then vigorously agitated for 1 min at room temperature and centrifuged at 4,000 × g for 2 min. The radioactivity in each layer was measured by liquid scintillation counting. Quenching corrections were made by the internal standard method.

In vitro Binding Assays

^{3}H-5-HT. The method of Bennett and Snyder (1976) was slightly modified as recently described (Nelson et al., 1978). The main modification consisted of incubating the membranes (a lysed P_2 fraction) at 37° C for 10 min (without pargyline and ascorbic acid) in the course of their preparation for the binding assays. This supplementary step eliminated most of the endogenous 5-HT trapped in membranes which usually interferes with the binding of ^{3}H-5-HT (see Bennett and Snyder, 1976; Nelson et al., 1978). ^{3}H-5-HT (0.5–4.6 nM) was incubated with 2 ml of the membrane preparation (corresponding to 0.25–0.85 mg prot.) for 7 min at 37° C after which the membranes were collected and washed on Whatman GF/B filters under vacuum. The filters were then placed in 10 ml of a counting solution (Aquasol, New England Nuclear) for determination of radioactivity by liquid scintillation spectrometry. The non-specific binding of ^{3}H-5-HT corresponded to that persisting in the presence of 10 µM "cold" 5-HT.

^{3}H-Spiroperidol and ^{3}H-Haloperidol. Striatal membranes were prepared as described for the binding of ^{3}H-5-HT except that the final suspension was made according to the conditions described by Creese and Snyder (1978) and contained the following: Tris-HCl (0.05 M), pargyline (10 µM), ascorbic acid (5.7 mM), NaCl (120 mM), KCl (5 mM), $CaCl_2$ (2 mM) and $MgCl_2$ (1 mM) at pH 7.4. ^{3}H-spiroperidol (0.048–0.426 nM) was incubated with 2 ml of the membrane preparation for 45 min at 37° C. ^{3}H-haloperidol (0.50–3.10 nM) was incubated with 2 ml of the membranes for 10 min at 37° C. In both cases the membranes were collected by centrifugation (8,000 × g, 30 min, 4° C) and washed once with 5 ml of ice-cold Tris-HCl-saline buffer (composition as above). After a second centrifugation, the pellet was finally sonicated in 1 ml of water. The radioactivity in a 0.5 ml aliquot of the suspension was measured by liquid scintillation counting. Non-specific binding of either ^{3}H-spiroperidol or ^{3}H-haloperidol was defined as that which occurred in the presence of 1 µM (+)-butaclamol. Using this centrifugation technique for stopping binding assays, the radioactivity finally entrapped by the membranes was similar whether 1 µM (+)-butaclamol, 1 µM spiroperidol or 1 µM haloperidol was included in the incubation mixture. In contrast, using the filtration technique (see Creese and Snyder, 1978; Leysen et al., 1978), we observed that the non-specific binding of ^{3}H-spiroperidol was 25–35% higher in the presence of 1 µM butaclamol or haloperidol than with 1 µM spiroperidol. As revealed by experiments without membranes, this resulted from the displacement by spiroperidol but not by the two other neuroleptics of the radioactivity entrapped by the GF/B filters (not shown). Such

Table 1. Comparison of the properties of methiothepin and spiroperidol as 5-HT antagonists. (A) ^{3}H-5-HT binding in lysed P_2 fractions from rat forebrain was measured with 1.5 nM of the labelled ligand and 9 different concentrations (10^{-9} M – 10^{-5} M) of either methiothepin or spiroperidol. IC 50 and *m* (the slope of the curve, see Nelson et al., 1978) were calculated by linear regression of logit-log inhibition plots. Each value is the mean ± SEM of 3 independent experiments. (B) 5-HT-sensitive adenylate cyclase in colliculi homogenates from new born rats (see Enjalbert et al., 1978) was measured with 10 μM 5-HT and 5 different concentrations (10^{-7} M – 10^{-5} M) of either methiothepin or spiroperidol. IC 50 and *m* were calculated as in A. Each value is the mean of two independent determinations. (C) The partitioning of ^{3}H-methiothepin and ^{3}H-spiroperidol between octanol and Tris-HCl buffer (pH 7.4) was performed as described in "Methods". Each value is the mean ± SEM of triplicate determinations (in two independent experiments) of the ratio of the radioactivity found in octanol to that found in the aqueous layer

Antagonist	A ^{3}H-5-HT binding		B 5-HT-sensitive adenylate cyclase		C Partition coefficient
	IC 50 (μM)	*m*	IC 50 (μM)	*m*	
Methiothepin	0.31 ± 0.07	0.62 ± 0.05	3.95	0.74	87.8 ± 2.5
Spiroperidol	0.34 ± 0.08	0.35 ± 0.09	11.83	0.39	109.8 ± 3.1

observations led us to choose the centrifugation technique for the measurements of ^{3}H-spiroperidol and ^{3}H-haloperidol binding.

^{3}H-Methiothepin. In most cases the preparation of membranes used for the binding of ^{3}H-methiothepin in vitro was the same as described above for the binding of ^{3}H-5-HT. In addition, two methods of tissue fractionation were used in order to further analyze the subcellular distribution of ^{3}H-methiothepin binding. According to Cotman and Matthews (1971), the following fractions were obtained: P_1 (crude nuclear fraction), P_2 (crude mitochondrial fraction), S_2 (17,000 × g supernatant corresponding to P_2), myelin, B (purified synaptosomal fraction), M_1 (purified mitochondria), $F_1 + F_2$ (fractions at the top of the sucrose gradient mainly containing synaptic plasma membranes). The second method was that of Laduron et al. (1975) which allows the fractionation of brain tissues into a nuclear (N), a heavy mitochondrial (M), a light mitochondrial (L), a microsomal (P) and finally a soluble (S) fraction.

The incubation of the membrane preparations with ^{3}H-methiothepin and other unlabelled compounds was generally carried out at 37° C for 10 min in 0.05 M Tris-HCl, pH 7.4, containing 130 mM NaCl. The incubation was stopped either by filtration (as for ^{3}H-5-HT binding), or by centrifugation (as for ^{3}H-haloperidol and ^{3}H-spiroperidol binding). Since non-negligible amounts of ^{3}H-methiothepin (10 – 12 % of the total radioactivity in the sample) were found to adsorb to GF/B filters even after 3 × 5 ml washing with ice-cold Tris-HCl buffer, appropriate blanks (samples without membranes) were included under all conditions when using the filtration method. This was critical for accurate calculations since, in the absence of membranes, ^{3}H-methiothepin entrapped by the filter was displaceable by unlabelled methiothepin (in the presence of 0.1 mM of the unlabelled drug, the entrapped radioactivity decreased by 65 %). These observations led us to use the centrifugation method as often as possible to collect the membranes after the incubation with ^{3}H-methiothepin.

5-HT-Sensitive Adenylate Cyclase

The enzyme source was an isotonic homogenate of colliculi from new born rats as described in detail elsewhere (Bourgoin et al., 1977). The assay consisted of measuring the conversion of α^{32}-P-ATP into ^{32}P-cyclic AMP under the conditions of Enjalbert et al. (1978).

In vivo Administration of Methiothepin

^{3}H-methiothepin was administered in water (0.5 ml per rat) at a dose of 0.1 or 0.5 mCi/kg, i.p. Treatment with the unlabelled drug consisted of an i.p. injection of 20 mg/kg of methiothepin maleate as a fine suspension in water.

For the determination of the amount and distribution of radioactivity in the brain after the administration of ^{3}H-methiothepin, rats were killed at various times after the injections and the brain regions of interest were dissected and homogenized in 20 volumes of 0.05 M Tris-HCl, pH 7.4, using a Polytron PT 10 OD. The homogenate was centrifuged at 35,000 × g for 20 min and the supernatant (S_a) was decanted. The pellet (P_a) was resuspended in water, diluted 1:1 with 10 % trichloroacetic acid, and then centrifuged as above. The supernatant (S_b) was decanted and the pellet was vigorously agitated in 5 ml of chloroform:methanol (1:1 by vol.). After centrifugation (35,000 × g, 20 min), the supernatant (S_f) was carefully removed into glass counting vials and completely evaporated overnight in a chromatographic oven at 80° C. The pellet (P_f) was dried at 60° C for 30 min and finally dissolved in 1 ml of 1 N NaOH. Radioactivity was determined in all fractions by liquid scintillation spectrometry and corrected for quenching using the method of internal standards.

Endogenous levels of 5-HT in various brain areas were determined using the radioenzymatic method of Saavedra et al. (1973).

In order to avoid interfering substances (ascorbic acid, Tris) in their measurement with the folin-phenol reagent, proteins in the various media used in this study were first precipitated with 10 % trichloroacetic acid, collected by centrifugation and then dissolved in 1 N NaOH. The procedure of Lowry et al. (1951) was applied to this solution with bovine serum albumin (Sigma) as the standard.

Statistical calculations were made as described by Snedecor and Cochran (1967). When *P* was greater than 0.05 (Student's *t*-test), a difference was considered to be non-significant.

Results

Basis for the Choice of ^{3}H-Methiothepin as a Ligand of 5-HT Receptors: Comparison with ^{3}H-Spiroperidol

As shown in Table 1, methiothepin and spiroperidol inhibited both the binding of ^{3}H-5-HT to forebrain synaptosomal membranes and the stimulatory effect of 5-HT on adenylate cyclase activity in homogenates of colliculi from new born rats. For each drug, the IC 50 value for the inhibition of this adenylate cyclase was higher (by more than one order of magnitude) than that

characterizing their efficiency to displace ^{3}H-5-HT from its specific high affinity binding site. Although the interaction of each drug with 5-HT receptors seemed rather complex (the slopes *m* of logit-log inhibition curves were lower than 1.0 in all cases, Table 1), the data obtained, particularly the drugs' effects on 5-HT-sensitive adenylate activity, revealed that methiothepin was slightly more potent than spiroperidol as a 5-HT antagonist.

One important limitation in the use of labelled drugs as specific ligands of neurotransmitter receptors consists of their binding to nonreceptor sites. Since this non-specific binding increases with the lipophilic character of the ligand (see Martres et al., 1978), the partitioning of ^{3}H-methiothepin between octanol and aqueous layers was determined and compared to those of other ^{3}H-molecules already known as suitable ligands in binding assays. Although the partition coefficient of ^{3}H-methiothepin at neutral pH (see Table 1) was much higher than that of ^{3}H-haloperidol (18.9 ± 0.4) and ^{3}H-5-HT (0.010 ± 0.003), it was significantly lower than that of ^{3}H-spiroperidol (Table 1) indicating a lower lipophilic character than the latter drug.

In vitro Binding of ^{3}H-Methiothepin to Rat Brain Membranes

^{3}H-methiothepin bound very rapidly to various membrane preparations incubated in 0.05 M Tris-HCl buffer, pH 7.4, reaching a plateau in less than 3 min at 37° C. At this time, the radioactivity trapped per mg of membrane protein (lysed P_2 fraction from the whole forebrain) represented 45–55% of the total radioactivity in the sample. This percentage did not change significantly when the concentration of ^{3}H-methiothepin was varied between 0.1 nM and 60 nM. In order to check whether this binding was at least partly "specific" i.e. if "cold" methiothepin was able to displace bound ^{3}H-methiothepin, samples were incubated with 0.50 nM of the labelled ligand in the presence of various concentrations of "cold" methiothepin. An inhibition of ^{3}H-methiothepin binding first occurred with 10 µM (11%) and reached 26% and 51% in the presence of 0.1 mM and 1 mM of the unlabelled molecule respectively. Higher concentrations of "cold" drug could not be used owing to the poor solubility of methiothepin in the medium used for binding assays. Since the displacement of bound ^{3}H-methiothepin was not leveling off even with 1 mM of cold methiothepin, the "specific" component of the binding was extremely difficult to estimate. Further attempts were then made to increase the part of bound ^{3}H-methiothepin displaceable by the cold drug. As shown in Fig. 1, lowering the pH of the incubating

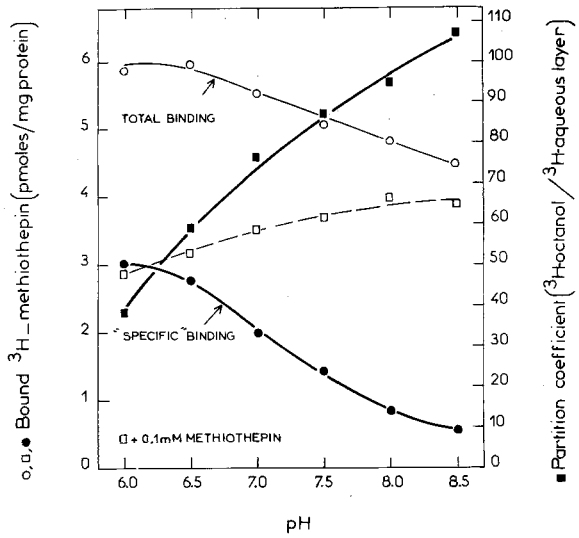


Fig. 1. pH dependent changes in the binding of ^{3}H-methiothepin to forebrain membranes. Comparison with the partitioning of ^{3}H-methiothepin between octanol and aqueous layers. Forebrain membranes (corresponding to 0.96 mg of proteins) in 2 ml of 0.05 M Tris-HCl adjusted to various pH's were incubated at 37° C for 10 min with 4.80 nM of ^{3}H-methiothepin in the presence (□) or the absence (○) of 0.1 mM unlabelled methiothepin. ^{3}H-methiothepin bound to membranes was then measured by the filtration method (see the text). Each point is the mean of triplicate determinations of total binding (○), non-specific binding (□, persisting in the presence of 0.1 mM of "cold" methiothepin) and of the difference between these two values (●, the so-called "specific" binding). Bound ^{3}H-methiothepin is expressed in pmoles of the labelled ligand per mg of membrane protein. At pH 7.5 for instance, these values correspond to: total binding: 8,762 ± 257 cpm/sample; non specific binding: 6,412 ± 176 cpm/sample with a total radioactivity of 19,114 ± 158 cpm of ^{3}H-methiothepin (means ± SEM of triplicate determinations) in 2 ml of incubating medium. Partitioning of ^{3}H-methiothepin between octanol and aqueous layers was performed as described in "Methods". Each point (■) is the mean of triplicate determinations of the partition coefficient (see the definition in the legend to Table 1)

mixture resulted in an increased efficiency of "cold" methiothepin to displace the bound ^{3}H-ligand. A parallel study on the partitioning of labelled methiothepin between octanol and aqueous layers revealed that the lipophilic character of the molecule increased regularly as a function of pH (Fig. 1). These findings further confirmed that the binding of a labelled molecule to non-specific sites is directly related to its lipophilic properties (see Martres et al., 1978).

The effects of ions (Ca^{2+}, Mg^{2+}, Na^+, K^+, Cl^-, $H_2PO_4^-$, HPO_4^{2-}) on ^{3}H-methiothepin binding to a lysed P_2 fraction from the forebrain revealed that Na^+ slightly improved the efficiency of 0.1 mM methiothepin to displace the bound ligand (the inhibition by the "cold" molecule increased from 26–36% when the concentration of Na^+ in the incubating mixture was raised from 0–130 mM). Accordingly, in order to increase the specific component of ^{3}H-methiothepin

Table 2. Effects of various drugs on ^{3}H-methiothepin binding in a lysed P_2 fraction from the rat forebrain. The lysed P_2 fraction was incubated for 10 min at 37° C with 4.47 nM ^{3}H-methiothepin and another "cold" drug when indicated. Bound ^{3}H-methiothepin is expressed in pmoles per mg protein. Each value is the mean ± SEM of quadruplicate determinations. The other tested compounds, pargyline, ascorbic acid, dithiothreitol and β-mercaptoethanol, were devoid of any effect even at 1 mM

Drug		Bound ^{3}H-methiothepin	%
–		5.20 ± 0.10	100
Methiothepin	0.1 mM	3.34 ± 0.07	64
Promethazine	0.1 mM	4.00 ± 0.08	77
Promethazine + Methiothepin	0.1 mM + 0.1 mM	3.21 ± 0.07	62
Metergoline	0.1 mM	3.86 ± 0.12	74
Chlorpromazine	0.1 mM	4.02 ± 0.15	77
Haloperidol	0.1 mM	4.16 ± 0.10	80
d-LSD	0.1 mM	4.80 ± 0.10	92
(+)-butaclamol	0.1 mM	4.78 ± 0.05	92
Dopamine	0.1 mM	4.84 ± 0.09	93
Serotonin	0.1 mM	5.25 ± 0.16	101

binding at a physiological pH, assays generally consisted of incubating membranes in 2 ml of 0.05 M Tris-HCl, pH 7.4, containing 130 mM NaCl. For convenience, the time of incubation at 37° C was fixed at 10 min.

Under these conditions, the radioactivity which remained associated with the membranes was readily extracted by chloroform:methanol (1:1). Thin layer chromatography performed as described in "Methods" demonstrated that ^{3}H-methiothepin was the only radioactive compound in this organic extract.

In addition to methiothepin, several drugs (promethazine, metergoline, chlorpromazine, haloperidol) also slightly reduced the binding of ^{3}H-methiothepin to forebrain membranes (Table 2). As illustrated in Table 2, the binding component affected by promethazine was likely the same as that inhibited by unlabelled methiothepin since promethazine did not further reduce ^{3}H-methiothepin binding when 0.1 mM methiothepin was added to the assay mixture. In contrast, *d*-LSD, (+)-butaclamol, DA and 5-HT hardly altered ^{3}H-methiothepin binding (Table 2).

When the total binding of ^{3}H-methiothepin was examined in various areas of the rat brain, no large regional differences were seen (Table 3). However, the so-called "specific" binding component which was displaceable by 0.1 mM methiothepin, was larger (by about 50 %) in the striatum and the cerebral cortex than in the cerebellum (Table 3). As shown in Table 3, no correlation existed between this binding component

Table 3. Regional distribution of ^{3}H-methiothepin binding in vitro in the CNS of the adult rat. Lysed P_2 fractions of various regions of rat brain were incubated for 10 min at 37° C with 1.80 nM ^{3}H-methiothepin. "Specific" binding represents the amount of bound ^{3}H-methiothepin displaceable by 0.1 mM methiothepin. Bound (total and "specific") ^{3}H-methiothepin is expressed in pmoles per mg protein. Each value is the mean ± SEM of quadruplicate determinations. 5-HT levels (mean ± SEM of 8 determinations) are expressed in μg per g of fresh tissue

Brain region	5-HT	Bound ^{3}H-methiothepin	
		Total	"Specific"
Cerebral cortex	0.28 ± 0.03	1.98 ± 0.05	0.74 ± 0.03
Striatum	0.49 ± 0.03	1.98 ± 0.07	0.76 ± 0.03
Olfactory tubercle	0.60 ± 0.05	2.05 ± 0.02	0.63 ± 0.02
Brain stem	0.72 ± 0.04	1.81 ± 0.09	0.58 ± 0.02
Hippocampus	0.45 ± 0.04	1.84 ± 0.06	0.62 ± 0.03
Cerebellum	0.08 ± 0.01	1.73 ± 0.05	0.51 ± 0.03

and 5-HT levels in the 6 brain areas examined ($r = 0.167$).

Attempts to isolate a fraction with a higher specific binding capacity than that of the crude synaptosomal fraction (P_2) by the method of Cotman and Matthews (1971) were unsuccessful. For all the fractions examined (see "Methods"), only 20–39 % of bound ^{3}H-methiothepin was displaced by 0.1 mM of unlabelled methiothepin. Similar results were found when the subcellular fractionation of rat forebrain was performed as described by Laduron et al. (1975) (data not shown).

Time Course of ^{3}H-Methiothepin Elimination from Brain After Its in vivo Administration

The kinetics of ^{3}H-methiothepin disposition in brain was examined to estimate the amount of the drug present in tissues at various times after its peripheral administration.

As early as 1 h after the intraperitoneal injection of ^{3}H-methiothepin (500 μCi/kg), only 1 – 1.5 ‰ or 0.1 – 0.15 % of the injected radioactivity was found in the whole brain. A regional study did not reveal significant differences between the various structures examined (cerebral cortex, striatum, olfactory tubercle, hippocampus, brain stem and cerebellum). The co-administration of unlabelled methiothepin (20 mg/kg i.p.) with the labelled ligand only resulted in a slight reduction (10 – 18 %) in the accumulation of the radioactivity in brain tissues (data not shown).

The extraction of radioactivity which had accumulated in brain tissues 1 h after the administration of ^{3}H-methiothepin indicates that about 70 % of the ^{3}H was membrane-bound (see Table 4 for the striatum for instance). Only 23 % of the bound radioactive material

Table 4. Subcellular distribution of radioactivity in the striatum after in vivo treatment with ^{3}H-methiothepin. ^{3}H-methiothepin (500 µCi/kg i.p.) was administered with or without 20 mg/kg methiothepin to groups of 4 adult rats. Animals were killed 1 h later and tissue fractionation of their striata was performed as described in "Methods". Each value is the mean ± SEM of 4 determinations of ^{3}H expressed in nCi per g of fresh tissue. Figures in the right column correspond to the ratio (in per cent) of ^{3}H found in each fraction from animals treated with "cold" methiothepin to that occurring when only ^{3}H-methiothepin was injected

^{3}H	−	+ methiothepin	%
Homogenate	95.5 ± 7.7	78.1 ± 6.1	81.7
P_a	67.6 ± 5.1	46.1 ± 2.3	68.2
S_a	25.7 ± 1.4	35.4 ± 1.4	137.7
S_b	15.5 ± 1.0	13.4 ± 1.2	86.5
S_f	48.6 ± 2.3	32.6 ± 4.1	67.1
P_f	1.8 ± 0.3	1.5 ± 0.5	83.3

was removed by precipitating proteins with trichloroacetic acid (Table 4). Indeed, most bound ^{3}H (70–75%) was associated with materials extracted in chloroform:methanol (1:1 by vol.). Thin layer chromatography of this organic extract indicated that about 40% of the radioactivity corresponded to authentic ^{3}H-methiothepin. Accordingly, at 1 h after the i.p. administration of 20 mg methiothepin per kg body weight, the concentration of authentic methiothepin in the whole brain did not exceed 3.8 µM.

A time-course study of ^{3}H disappearance from brain tissues after ^{3}H-methiothepin administration revealed complex kinetics. As illustrated in Fig. 2 for the hippocampus, the half-life of ^{3}H in whole tissues during the initial period was equal to about 1 day and only 8 h in the Pa fraction (membranes sedimenting at 35,000 × g). From these curves (Fig. 2) and from TLC analysis of ^{3}H-material accumulated in tissues, it was estimated that the concentration of the drug bound to membranes in the hippocampus remained higher than 0.3 µM (i.e. higher than the IC 50 value for ^{3}H-5-HT binding, see Table 1) for the 10–12 h following the i.p. injection of 20 mg methiothepin per kg of body weight.

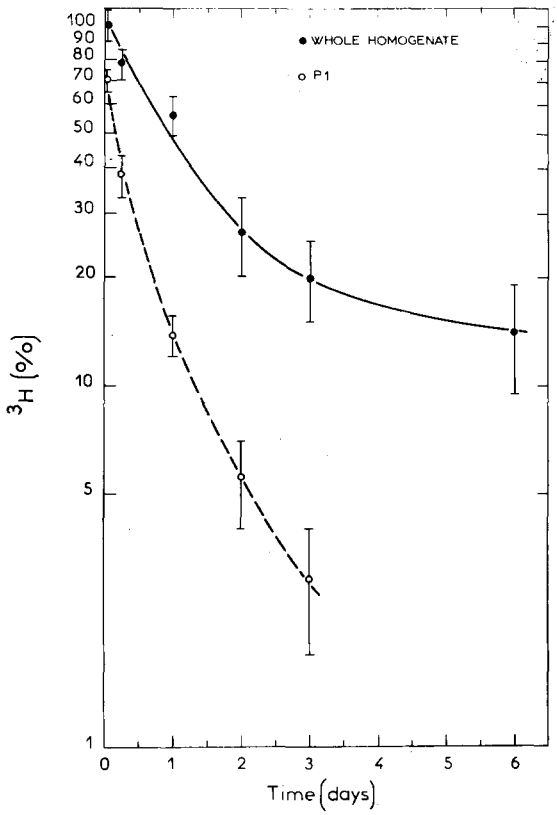


Fig. 2. Time course of ^{3}H disappearance in the hippocampus following the i.p. administration of ^{3}H-methiothepin to rats. ^{3}H-methiothepin (100 µCi/kg) was administered on the 6th, 3rd, 2nd or 1st day or 6 h or 1 h before sacrifice. The radioactivity accumulated in the homogenate and P_a fraction of the hippocampus was measured as described in "Methods" and expressed in per cent of that found in the homogenate (corresponding to 18.4 ± 1.7 nCi/g of fresh tissue) from rats killed 1 h after the treatment. Each point is the mean ± SEM of 4 determinations

Effects of the in vivo Administration of Methiothepin on the High Affinity Binding of ^{3}H-5-HT to Synaptosomal Membranes

As illustrated in Fig. 3, the administration of methiothepin (20 mg/kg i.p.) 1 h before death, significantly reduced the capacity of forebrain membranes to bind ^{3}H-5-HT to specific high affinity sites. One day after the treatment, when the concentration of methiothepin still bound to tissues should be less than 0.15 µM (as estimated from the time course of ^{3}H-disappearance after ^{3}H-methiothepin treatment and TLC), the methiothepin-induced inhibition of ^{3}H-5-HT binding was no longer seen. Later, i.e. on the 3rd and 6th days following the injection, a significant increase in the ^{3}H-5-HT binding capacity was detected (Fig. 3).

A similar time-course of changes in ^{3}H-5-HT binding was observed in synaptosomal membranes from the hippocampus and the striatum (the two regions containing the highest densities of ^{3}H-5-HT specific binding sites, see Nelson et al., 1978). However, significant increases were noted on the 2nd and the 3rd days after the treatment only in the hippocampus (Table 5).

Scatchard analyses of ^{3}H-5-HT binding to membranes from the forebrain, the striatum and the hippocampus revealed that both the significant decreases and increases occurring after methiothepin treatment were associated with fluctuations in the number of binding sites without any change of the dissociation constant. This is illustrated in Fig. 4 which shows the

selective increase in the concentration of specific binding sites for ^{3}H-5-HT in hippocampal membranes on the 3rd day after methiothepin administration.

Effect of the in vivo Administration of Methiothepin on the Characteristics of Specific Binding Sites for ^{3}H-Haloperidol and ^{3}H-Spiroperidol on Striatal Membranes

As methiothepin has also been shown to block dopaminergic receptors (Enjalbert et al., 1978; Lloyd and Bartholini, 1974), the binding of ^{3}H-haloperidol and ^{3}H-spiroperidol was estimated at various times after its peripheral administration (20 mg/kg i.p.).

At a time when ^{3}H-5-HT binding capacity was maximally increased, i.e. on the 3rd day following methiothepin treatment, no significant alteration in ^{3}H-haloperidol binding was detected in striatal membranes (control: Kd = 2.1 ± 0.3 nM, B_{max} = 0.56 ± 0.08 pmoles/mg protein, means ± SEM of 3 independent experiments; methiothepin-treated: Kd = 2.4 ± 0.1 nM, B_{max} = 0.62 ± 0.11 pmoles/mg protein, n = 3). Similar findings were obtained with ^{3}H-spiroperidol as the labelled ligand (control:

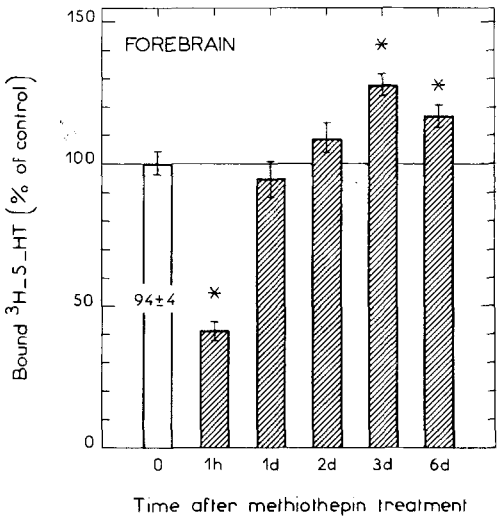


Fig. 3. Time course of changes in the specific binding of ^{3}H-5-HT in the forebrain of methiothepin-treated rats. Groups of 5 rats were killed 6, 3, 2, 1 day or 1 h after the i.p. administration of methiothepin (20 mg/kg). Binding of ^{3}H-5-HT to a lysed P_2 fraction from the forebrain was measured with 1.9 nM of the labelled ligand. Each bar represents the mean ± SEM of 5 determinations in two independent experiments. The figure in the first bar corresponds to the absolute amount of ^{3}H-5-HT specifically bound (in fmoles/mg protein) to membranes from control rats. * $P < 0.05$ when compared to the control value

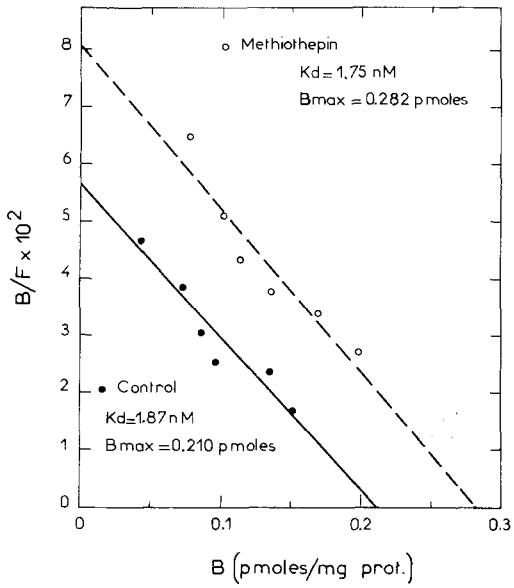


Fig. 4. Scatchard plots of ^{3}H-5-HT binding to hippocampal membranes from control and methiothepin-treated rats. Methiothepin (20 mg/kg i.p.) or saline was administered 72 h before death. Lysed P_2 fractions from the hippocampi were incubated with 0.49, 0.98, 1.46, 1.95, 2.93 or 4.61 nM of ^{3}H-5-HT as described in "Methods". Each point is the mean of quadruplicate determinations. B_{max} are expressed in pmoles ^{3}H-5-HT bound per mg membrane protein

Table 5. Effects of a single injection of methiothepin on the specific binding of ^{3}H-5-HT in the hippocampus and the striatum. The same protocol as that described in the legend to Fig. 3 was used except that lysed P_2 fractions were prepared from the hippocampus or the striatum. The specific binding of ^{3}H-5-HT was measured as described in "Methods" with 1.25 nM of the labelled ligand. Each value is the mean ± SEM of quadruplicate determinations (in two independent experiments) of bound ^{3}H-5-HT expressed in fmoles per mg protein. Figures in parentheses correspond to the percent of respective control values

		Bound ^{3}H-5-HT			
		Hippocampus	(%)	Striatum	(%)
Control		120 ± 5	(100)	84 ± 3	(100)
Methiothepin –	1 hour	66 ± 4*	(55)	59 ± 3*	(70)
	– 1 day	138 ± 7	(115)	91 ± 7	(108)
	– 2 days	153 ± 3*	(128)	94 ± 5	(112)
	– 3 days	163 ± 14*	(136)	90 ± 3	(107)
	– 6 days	143 ± 4*	(119)	94 ± 2*	(112)

* $P < 0.05$ when compared to respective control values

$Kd = 0.11 \pm 0.02$ nM, $B_{max} = 0.53 \pm 0.08$ pmoles/mg protein, $n = 3$; methiothepin-treated: $Kd = 0.10 \pm 0.02$ nM, $B_{max} = 0.54 \pm 0.08$ pmoles/mg protein, $n = 3$).

Discussion

Several properties of methiothepin suggested that its tritiated derivative could be used for selective labelling of the putative "antagonist" form (see Bennett and Snyder, 1976) of central 5-HT receptors. Thus, in the two biochemical tests currently available, i.e., the measurements of ^{3}H-5-HT high affinity binding and of the 5-HT-sensitive adenylate cyclase, methiothepin was slightly more potent than spiroperidol as a 5-HT antagonist. Moreover, its lipophilic character was slightly less pronounced than that of spiroperidol suggesting a priori a lower binding at non-specific sites (see Martres et al., 1978; Fig. 1 of the present paper). However, in spite of extensive efforts, it was not possible to appreciate either in vitro or in vivo the proportion of ^{3}H-methiothepin binding occurring specifically to 5-HT receptors. Indeed, the irreducible non-specific binding was too high to allow quantitative studies. A small displacement of ^{3}H-methiothepin was only seen with high concentrations of unlabelled methiothepin and of drugs such as promethazine and haloperidol which are essentially blockers of histaminergic and dopaminergic receptors respectively suggesting that part of bound ^{3}H-methiothepin was also associated with these receptors. These findings were not surprising in light of the known anti-dopaminergic (Enjalbert et al., 1978; Lloyd and Bartholini, 1974) and anti-histamine (Protiva, 1977) effects of the drug.

The firm attachment of ^{3}H-methiothepin to membranes was well documented by the treatments required for its extraction from tissues after its in vivo administration or its in vitro incubation with lysed synaptosomal fractions. Indeed, protein precipitation with trichloroacetic acid only removed 20–25% of ^{3}H-material bound to membranes. Since most of the bound radioactivity could be extracted with chloroform:methanol (1:1 by vol.), ^{3}H-methiothepin may mainly be associated with the lipid portion of the membranes. This might explain why the "specific" part of ^{3}H-methiothepin binding to membranes (i.e. that bound to receptor proteins) was so discrete.

Nevertheless, the binding of methiothepin to specific 5-HT receptors was likely as strong as elsewhere in the membranes since extensive washings (in the course of the preparation of membranes) did not suppress the inhibition of ^{3}H-5-HT binding occurring 1 h after its in vivo administration. As already observed in vitro (Fillion et al., 1978; Nelson et al., 1978), the in vivo inhibition of ^{3}H-5-HT binding by methiothepin was (apparently) non-competitive (selective decrease in B_{max}) as a result of the very slow desorption of the drug from the 5-HT receptors. The estimation of the amount of methiothepin bound to membranes (2.7 μM in Pa fraction) 1 h after its administration was quite compatible with the extent of inhibition of ^{3}H-5-HT binding occurring at this time.

Kinetic analyses with ^{3}H-methiothepin suggested that the concentration of the drug bound to membranes in brain remained higher than 0.3 μM for the 10–12 h following the i.p. injection of 20 mg methiothepin per kg body weight. This led us to look for possible changes in ^{3}H-5-HT binding after a sustained occupancy of the specific sites by a potent antagonist. The present study clearly revealed that an acute treatment with a large dose (20 mg/kg i.p.) of methiothepin was sufficient to induce the appearance of new specific binding sites for ^{3}H-5-HT, particularly in the hippocampus. This hypersensitivity phenomenon developed quite rapidly since a significant increase in the number of binding sites for ^{3}H-5-HT was detected on the 2nd day after a single administration of the drug. This was in sharp contrast to previous findings showing an increase in the concentration of ^{3}H-5-HT binding sites in brain tissues only after chronic treatment with drugs such as haloperidol (Muller and Seeman, 1977) or p-chlorophenylalanine (Steigrad et al., 1978). The hypersensitivity induced by methiothepin seemed to be restricted to 5-HT receptors since no change in the binding characteristics of DA receptor ligands (^{3}H-haloperidol and ^{3}H-spiroperidol) could be detected. Interestingly enough, an increased number of binding sites for ligands of DA receptors after a single injection of classical neuroleptics has never been reported. However, a rapid development of a hypersensitivity phenomenon has been well described in behavioral studies and by measuring changes in the rate of DA turnover (Hyttel, 1977, 1978; Martres et al., 1977).

Another treatment which has resulted in the proliferation of new binding sites for ^{3}H-5-HT in the CNS consisted of selective lesioning of serotoninergic innervation by intracerebral injection of 5,7-dihydroxytryptamine (Nelson et al., 1978). Although this "supersensitivity phenomenon" develops more slowly than that produced by methiothepin treatment, it also preferentially occurs in the hippocampus. Marked regional differences in the development of supersensitivity have been already described in the case of dopaminergic (Friend et al., 1978; Hitri et al., 1978; Kaneno et al., 1978) and adrenergic (Dismukes and Daly, 1976) receptors. The present findings therefore confirm and extend this conclusion to 5-HT receptors in the CNS.

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