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[^{3}H]Tetrahydrotrazodone Binding

Association with Serotonin$_1$ Binding Sites

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SUMMARY

High (17 nM) and low (603 nM) affinity binding sites for [^{3}H]tetrahydrotrazodone ([^{3}H]THT), a biologically active analogue of trazodone, have been identified in rat brain membranes. The substrate specificity, concentration, and subcellular and regional distributions of these sites suggest that they may represent a component of the serotonin transmitter system. Pharmacological analysis of [^{3}H]THT binding, coupled with brain lesion and drug treatment experiments, revealed that, unlike other antidepressants, [^{3}H]THT does not attach to either a biogenic amine transporter or serotonin$_2$ binding sites. Rather, it would appear that [^{3}H]THT may be an antagonist ligand for the serotonin$_1$ binding site. This probe may prove of value in defining the mechanism of action of trazodone and in further characterizing serotonin receptors.

INTRODUCTION

Membrane binding assays have proven useful for identifying the site of action for a variety of hormones and drugs (1). By characterizing their specific binding site this approach has made it possible to understand better the actions of these agents at both the cellular and molecular levels (2, 3). Furthermore, these procedures have been used to identify endogenous ligands for some drug receptor sites (4).

Antidepressants have also been studied in this manner (5). [^{3}H]Mianserin, [^{3}H]doxepin, [^{3}H]imipramine, and [^{3}H]desipramine are some of the ligands used and, in all cases, the binding component has ultimately been found to be a neurotransmitter receptor or transport site (6–9). Thus, [^{3}H]mianserin labels both 5-HT$_2$[5] and histamine receptors; [^{3}H]doxepin attaches to the histamine$_1$ receptor; [^{3}H]imipramine and [^{3}H]desipramine appear to bind to a portion of the synaptic transport sites for serotonin and norepinephrine, respectively.

In recent years a number of chemically diverse substances have been found to possess antidepressant activity (10). Among these is trazodone (Desyrel), a triazolopyridinone (11). Clinical trials have revealed that this substance is an effective broad-spectrum antidepressant (12, 13). Although reports suggest that trazodone may be influencing primarily serotonergic activity, its precise mechanism is unclear since the drug both stimulates and inhibits this neurotransmitter system (14–16). Moreover, trazodone is known to be an *alpha*-adrenergic blocker, an action that is most likely related to its sedative effects rather than its antidepressant effects (17). Unlike the tricyclics, trazodone has no anticholinergic activity (18).

The present study was undertaken to define the locus of action of trazodone. Using [^{3}H]THT, a biologically active analogue, a saturable and specific binding site has been identified having characteristics suggesting that this substance is a ligand for brain serotonin receptors.

MATERIALS AND METHODS

[^{3}H]THT (Fig. 1) was prepared by tritium reduction of trazodone (New England Nuclear Corporation, Boston, Mass.). The specific activity (5.66 Ci/mmole) and radiochemical purity (>97%) were established by the manufacturer. Thin-layer chromatography using silica gel and a chloroform/methanol/ammonium hydroxide (95:4.5:0.5) solvent system indicated that the radioligand was stable throughout the course of this study. Earlier experiments have revealed that the biological profile of unlabeled THT is similar to trazodone itself.[6] Thus, the doses of trazodone and THT found necessary to inhibit conditioned avoidance responses 50% were 41 and 66 mg/kg, respectively, whereas the potencies (IC$_{50}$) for inhibiting high-affinity serotonin transport were 0.5 μM and 4 μM, respectively (14).

Brain membranes were prepared from male Sprague-Dawley rat

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[5] The abbreviations used are: 5-HT, 5-hydroxytryptamine (serotonin); THT, tetrahydrotrazodone; *d*-LSD, *d*-lysergic acid diethylamide.

[6] L. A. Riblet, personal communication.

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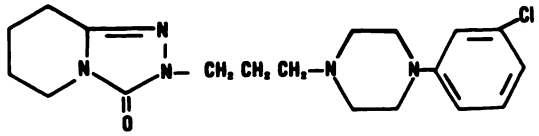

FIG. 1. *Chemical structure of THT*

(150–200 g) or calf brains. In the latter case the tissue was obtained from a local slaughterhouse at the time of death, transported to the laboratory in ice-cold saline, and then immediately dissected over ice into various regions. For the standard binding assay, brain tissue was homogenized in 15 volumes of 0.05 M Tris-citrate buffer (pH 7.0 at 4°), using a Brinkmann Polytron PT-10. The homogenate was centrifuged for 10 min at 15,000 × *g* (4°), the supernatant was decanted, and the tissue was rehomogenized as above. After a second centrifugation the pellet was resuspended in buffer to a concentration of approximately 0.6 mg of protein per milliliter. The binding assay was conducted in 2 ml of Tris-citrate buffer containing tissue (0.5–1.0 mg of protein) and 9 nM [^{3}H]THT. The samples were incubated for 30 min at 4° and the reaction was terminated by pouring the samples over glass-fiber filters (GF/B) that had been prewashed with incubation buffer containing 0.01% bovine serum albumin. After rinsing with 12 ml of cold buffer the filters were placed into scintillation vials containing 10 ml of Aquasol (New England Nuclear Corporation) and were then agitated for 1 hr at room temperature. Radioactivity was quantified by liquid scintillation spectrometry (Searle Mark III). Specific binding was defined as the amount of [^{3}H]THT displaced by 5 μM unlabeled THT or metergoline, and was routinely found to be approximately 50–60% of the total counts bound. Chromatographic analysis of isotope previously bound to brain membranes indicated no alteration of the radioligand during the incubation.

For subcellular binding studies the whole homogenate particulate was that fraction resulting from a 48,000 × *g* centrifugation (10 min) of a 15-volume 0.32 M sucrose homogenate of whole brain. The crude nuclear fraction (P_1) was the pellet obtained after a 10-min centrifugation of the whole brain homogenate at 1,000 × *g*. The crude synaptic membrane fraction (P_2) was the pellet derived from a 20,000 × *g* (20-min) centrifugation of the supernatant obtained in preparing the P_1. The microsomal fraction (P_3) was the pellet derived from a 48,000 × *g* centrifugation (20-min) of the supernatant resulting from the preparation of the P_2.

Rat brain cerebral cortical serotonin$_2$ binding was analyzed using a previously published procedure (19). For this assay, 0.3 nM [^{3}H]spiroperidol (29 Ci/mmole) (New England Nuclear Corporation) was used as a ligand, with a 100 μM serotonin blank. Synaptosomal high-affinity serotonin uptake was measured using the method of Coyle and Snyder (20). Electrolytic lesions of the midbrain raphe nucleus were made according to the method of Kuhar *et al.* (21).

For chronic antidepressant studies, rats received imipramine (10 mg/kg, i.p.) once daily for 21 consecutive days. Eighteen to twenty-four hours after the last injection the animals were decapitated, and the brains were rapidly removed and dissected over ice. The tissue was stored at −20° until assayed.

To study the pharmacological specificity of [^{3}H]THT binding, the standard assay conditions were used and the potencies of various agents to inhibit specific binding were determined by incubating the tissue with six to eight different concentrations of test compound. Log-probit analysis of the displacement data yielded the concentration required to inhibit 50% of the specifically bound radioligand (IC_{50}).

Drugs used in this study were obtained from the following sources: serotonin, norepinephrine, and dopamine from Sigma Chemical Company (St. Louis, Mo.); *d*-LSD from the National Institute on Drug Abuse; methysergide and clozapine from Sandoz; cyproheptadine, MK212, and metergoline from Merck Sharp & Dohme; cinanserin from Squibb; spiroperidol from Janssen; imipramine from CIBA; desipramine from USV Pharmaceuticals; fluoxetine from Eli Lilly; naloxone from Endo; chlorpromazine and cimetidine from Smith Kline & French; doxepin from Pfizer. All other agents were obtained from Mead Johnson.

RESULTS

Properties of [^{3}H]THT binding. Preliminary experiments indicated specific [^{3}H]THT binding reached equilibrium within 20 min and was linear with tissue concentrations up to 2 mg of protein. Specific binding was also studied as a function of temperature and pH in order to optimize the assay more fully. The binding was found to be greatest at 4°, being sone 25% lower at both 25° and 37°, and was highest between pH 7.0 and 7.2, declining considerably as the pH increased. Thus, at pH 8.0, [^{3}H]THT binding was only 35% of that observed at pH 7.0.

The binding site was characterized further by incubating brain membranes with 9 nM [^{3}H]THT in the presence and absence of various concentrations of unlabeled THT to define the blank (Fig. 2). A saturation binding assay was then conducted whereby the tissue was incubated with a variety of concentrations of [^{3}H]THT, with and without 5 μM unlabeled THT. The results of this assay revealed a high-affinity component having an apparent dissociation constant (K_d) of 17 nM and a lower-affinity site with a K_d of 603 nM. The concentrations (B_{max}) of the high- and low-affinity sites were estimated at 418 fmoles/mg of protein and 5.2 pmoles/mg of protein, respectively (Fig. 3A).

Association and dissociation experiments were performed to define more accurately the K_d of the higher-affinity site. The association rate (K_1) was calculated as 1.78×10^5 M^{-1} sec^{-1} and the dissociation rate (K_{-1}) as 1.53×10^{-3} sec^{-1}. Using these values, an equilibrium binding constant of 8.6 nM was found.

A significant amount of specific [^{3}H]THT binding was also detected in rat liver membranes. Saturation analysis indicated two components for this site, one having a K_d of 54 nM with a B_{max} of 641 fmoles/mg of protein, and the second having an apparent K_d of 1.7 μM with a B_{max} of 4.1 pmoles/mg of protein (Fig. 3B).

Brain regional and subcellular distribution of [^{3}H]THT binding. Based on the foregoing results the assay conditions used in subsequent experiments entailed incubating 0.5–1 mg of tissue protein for 30 min at 4° in 0.05 M Tris-citrate buffer (pH 7.0 at 4°) containing 9 nM [^{3}H]THT in the presence and absence of either 5 μM THT or metergoline (blank).

Assays conducted on various subcellular constitutents derived from whole rat brain revealed that the binding was most enriched in the crude synaptic membrane component. Binding in this fraction was approximately twice as great as that found in either the whole homogenate or in the crude nuclear fraction (P_1) (data not shown).

[^{3}H]THT binding was also found to be heterogeneously distributed throughout the calf brain (Table 1), being highest in the caudate nucleus and frontal cortex; intermediate in hippocampus, globus pallidus, putamen, and medullary tegmentum; and lowest in cerebellar regions. Identical results were obtained using either 5 μM THT or 5 μM meterogoline as blanks. A 2.5-fold range in binding was found among these 16 regions, with binding in white matter (corpus callosum) being only one-fifth of that detected in the caudate nucleus. Specific binding was found to be completely destroyed by boiling the brain membranes for 30 min prior to assay.

Pharmacological specificity of [^{3}H]THT binding. The

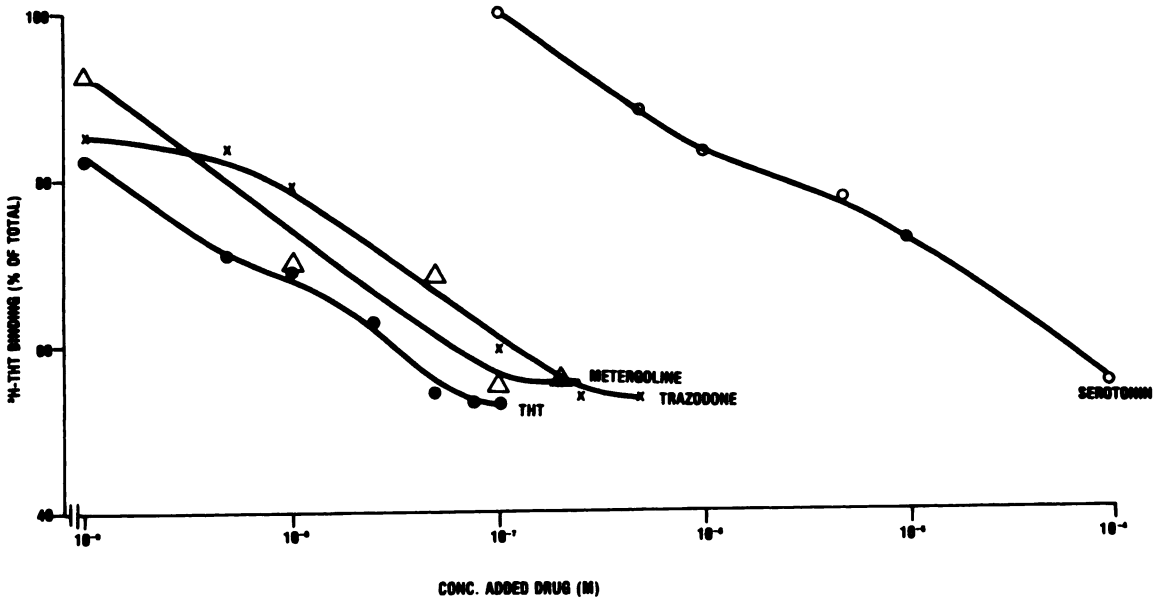

FIG. 2. *Displacement curves for [^{3}H]THT binding*

Membrane fractions (1.0 mg of protein) from rat brain homogenates were incubated in 0.05 M Tris-citrate (pH 7.0 at 4°) buffer containing various concentrations of [^{3}H]THT in the presence and absence of various concentrations of the indicated compounds. Values are the means of three separate experiments, each performed in triplicate.

potencies of various drugs and neurotransmitters to displace specifically bound [^{3}H]THT from cerebral cortical membranes were analyzed (Fig. 2; Table 2). Of the neurotransmitters tested, serotonin appeared to be the most potent, having an IC_{50} of 10 μM. Apart from THT itself, the next most potent drug was metergoline, a serotonin receptor antagonist, with an IC_{50} of 29 nM. The IC_{50} for trazodone was found to be 50 nM, some 3 times less than THT. All of these agents displaced the same total amount of [^{3}H]THT in brain membranes (Fig. 2). The antipsychotics spiroperidol and clozapine, with IC_{50} values in the 30–65 nM range, were also active at this site, as were the serotonin antagonists cinanserin and cyproheptadine,

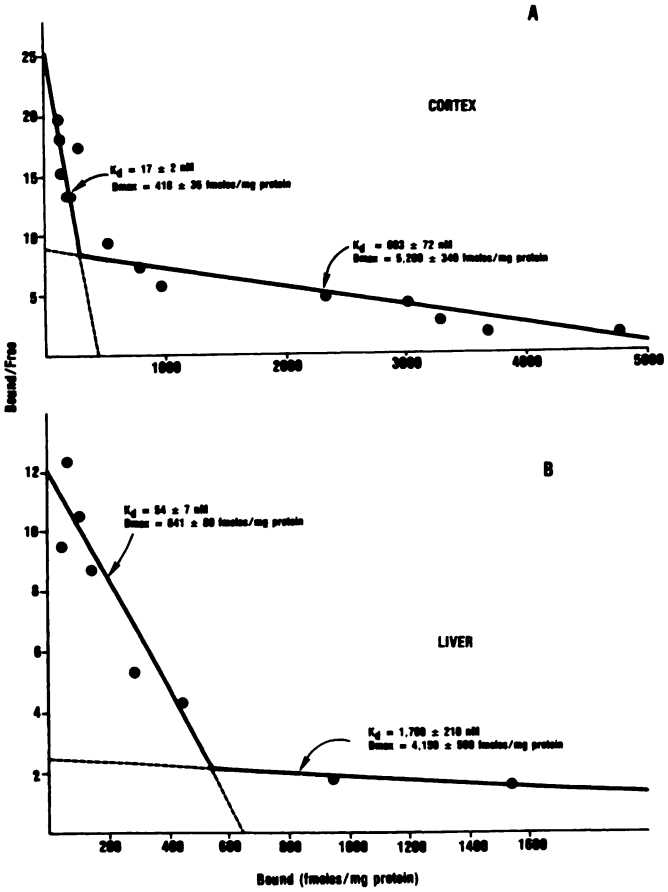

FIG. 3. *Rosenthal plot of specific [^{3}H]THT binding in rat membranes derived from cerebral cortex and liver homogenates.*

Values represent the means ± standard error of the mean of four to six separate experiments.

TABLE 1

Regional distribution of [^{3}H]THT binding in calf brain

The binding assay was performed on whole homogenate particulate fractions of various regions of calf brain. The assay was conducted by incubating the tissue for 30 min with 9 nM [^{3}H]THT in the presence and absence of 1 μM unlabeled THT. Each value represents the mean ± standard error of the mean of three separate experiments, each performed in triplicate.

Brain region	Specifically bound [^{3}H]THT
	fmoles/mg protein
Caudate nucleus	123 ± 9
Frontal cortex	121 ± 9
Hippocampus	84 ± 11
Globus pallidus	83 ± 10
Putamen	82 ± 8
Medullary tegmentum	79 ± 12
Superior colliculus	75 ± 10
Lateral cerebellar hemisphere	72 ± 4
Basal pons	72 ± 10
Thalamus	71 ± 13
Substantia nigra	70 ± 11
Pontine tegmentum	68 ± 9
Caudal cerebellar vermis	68 ± 5
Pre- and postcentral gyrus	61 ± 9
Anterior cerebellar vermis	59 ± 8
Midbrain tegmentum	49 ± 4
Corpus callosum	24 ± 1

TABLE 2

Substrate specificity of [^{3}H]THT binding in rat brain cerebral cortex and liver

The inhibition of specific [^{3}H]THT binding to rat brain cortex and liver homogenate by various compounds was assayed using the standard procedure described under Materials and Methods. The concentration of each compound that inhibited specific radioligand binding 50% (IC_{50}) was determined by log-probit analysis of at least six different concentrations. Each value is the mean ± standard error of the mean of three to six separate determinations, each performed in triplicate. The absence of a value indicates that the substance was not analyzed.

Compound	IC_{50}	
	Cerebral cortex	Liver
	nM	*nM*
Triazolopyridinones		
Tetrahydrotrazodone	15 ± 2	40 ± 3
Trazodone	50 ± 4	90 ± 9
Etoperidone	140 ± 11	—
Neurotransmitters		
Serotonin	10,000 ± 1,650	100,000 ± 4,000
Norepinephrine	100,000 ± 12,500	1,200 ± 76
Dopamine	>1 mM	—
Serotonergic Agents		
d-LSD	600 ± 71	—
Methysergide	1,100 ± 135	—
Metergoline	28 ± 6	12,000 ± 1,400
Dihydroergotoxine	9,000 ± 1,100	—
M-Chlorophenylpiperazine	2,300 ± 350	—
Cinanserin	68 ± 8	—
Cyproheptadine	99 ± 12	—
MK-212	4,000 ± 780	—
Noradrenergic Agents		
Phentolamine	2,000 ± 350	4,500 ± 500
Prazosine	1,500 ± 88	—
WB-4101	1,300 ± 331	2,800 ± 190
Antidepressants		
Imipramine	300 ± 42	—
Desipramine	360 ± 29	—
Doxepin	130 ± 10	—
Fluoxetine	800 ± 144	—
Antipsychotics		
Spiroperidol	63 ± 8	—
Clozapine	30 ± 5	—
Chlorpromazine	>1 mM	400 ± 100

Compounds Inactive at 1 μM

Diazepam, cimetidine, naloxone, histamine, indalpine, oxypertine, indoramin, loxapine, nomifensine, pridefine, molindone, maprotiline, indomethacin, azepindole, benperidol, ketanserin, citalopram, pindolol, zometapine, amoxipine, zimelidine, quipazine, bupropion, pimozide, viloxazine, trebenzomine, pirandamine, clothiapine, iprindole, chlordiazepoxide, ICI 125, 211, phenobarbital, tryptophan, taurine, glycine, and GTP.

which had affinities of less than 100 nM. Numerous drugs had IC_{50} values within the 100–1000 nM range, including the antidepressants imipramine, desipramine, doxepin, and fluoxetine and the psychotomimetic *d*-LSD. Etoperidone, another triazolopyridinone, had similar activity. Less potent were methysergide, dihydroergotoxine, phentolamine, prazosine, WB-4101, and MK-212, all of which had IC_{50} values in the low micromolar range. A host of substances, including antidepressants (iprindole), anxiolytics (diazepam), antihistamines (ICI 125, 211) and amino acids (glycine), were much less active, causing no displacement of [^{3}H]THT at 1 μM.

With regard to [^{3}H]THT binding in rat liver membranes, trazodone and THT were the most potent of the compounds tested, having IC_{50} values of 90 and 40 nM, respectively. Unlike the brain binding site, norepinephrine was almost 100 times more potent than serotonin, having an IC_{50} of 1.2 μM. Moreover, metergoline was 500-fold weaker in inhibiting [^{3}H]THT binding in liver membranes than in brain, with an IC_{50} in liver of 12 μM, whereas chlopromazine was substantially more potent on the liver binding site than on brain (Table 2). Phentolamine and WB-4101 were equipotent in the two systems.

Effect of raphe lesion and imipramine treatment on [^{3}H]THT binding. In order to determine whether [^{3}H]THT binding represents attachment to the presynaptic serotonin transport site, the midbrain raphe nucleus was electrolytically destroyed, and 5-HT uptake and [^{3}H]THT and 5-HT_2 binding were analyzed in frontal cortex 3 weeks after surgery (Table 3). Although there was a 50% reduction in 5-HT accumulation at this time, neither [^{3}H]THT nor [^{3}H]spiroperidol binding was significantly modified.

Chronic (3-week) administration of imipramine (10 mg/kg, i.p.) once daily to rats causes a decrease in 5-HT_2 receptor binding in frontal cortex (22). To examine further whether [^{3}H]THT and [^{3}H]spiroperidol label a similar site, rats were treated chronically, and imipramine and cortical [^{3}H]THT and [^{3}H]spiroperidol binding were analyzed (Table 3). Although imipramine induced a 44% decline in 5-HT_2 binding, no significant change in [^{3}H]THT attachment was noted.

DISCUSSION

The major finding of this study was the discovery of a specific binding site for [^{3}H]THT in rat brain tissue. The concentration of these sites, as well as their temperature and pH dependencies, are similar to those reported for a variety of brain neurotransmitter receptors (23). Moreover, the subcellular distribution of high-affinity [^{3}H]THT binding, the sensitivity to heat denaturation, and the heterogeneous distribution in brain support the no-

TABLE 3

Effect of raphe lesion or imipramine treatment on 5-HT uptake and [^{3}H]THT binding in rat brain frontal cortex

For the lesion experiments, the midbrain raphe nucleus was electrolytically lesioned 3 weeks prior to analysis. Serotonin uptake was conducted in a crude synaptosomal preparation, and [^{3}H]THT and [^{3}H]spiroperidol bindings were assayed using the procedures described under Materials and Methods. For the imipramine experiment, rats received imipramine (10 mg/kg, i.p.) once daily for 3 weeks and were decapitated 18 hr after the last injection. Each value is the mean ± standard error of the mean of six to eight separate experiments, each performed in triplicate.

Treatment	Subjects	5-HT uptake	Specific binding	
			[^{3}H]THT	[^{3}H]Spiroperidol
		cpm/mg protein/5 min	*fmoles/mg protein*	
Raphe lesion	Sham	2451 ± 291	126 ± 10	81 ± 3
	Operated	1324 ± 165*	125 ± 11	87 ± 3
Imipramine	Control	—	112 ± 7	75 ± 8
	Treated	—	103 ± 10	42 ± 7*

* $p < 0.05$ compared with the corresponding control (two-tailed *t*-test).

tion that [^{3}H]THT attaches specifically to some component of the synaptic membrane. Although a significant amount of binding was detected in liver, the affinity constants and pharmacological profile of these sites differed markedly from those found in brain. This suggests that the central and peripheral [^{3}H]THT binding sites represent different membrane constituents.

Because both trazodone and THT are relatively potent inhibitors of high-affinity serotonin uptake into brain tissue (14), it was conceivable that the [^{3}H]THT binding was to a component of the serotonin transport system. To examine this, binding was assayed in cortical tissue obtained from animals after destruction of the dorsal raphe nucleus. Whereas the lesion reduced serotonin uptake by almost 50%, indicating a substantial reduction in serotonin input, [^{3}H]THT and [^{3}H]spiroperidol binding were unchanged, suggesting that neither was attaching to sites on serotonergic terminals.

The substrate specificity of high-affinity [^{3}H]THT binding indicates a possible association with brain serotonin receptors. Of the neurotransmitters studied, serotonin was the most potent as an inhibitor of [^{3}H]THT binding, being some 10 times more active than norepinephrine, and over 100 times greater than dopamine. Moreover, those agents known to interact with serotonin receptors were, as a group, more active than other drug classes. Most striking was the fact that metergoline, cinanserin, cyproheptadine, and spiroperidol—serotonin receptor antagonists—were the most potent compounds found. It is notable that the apparent affinity constants for this chemically diverse group of drugs are quite similar to those calculated for the triazolopyridinones (trazodone and etoperidone), indicating that the [^{3}H]THT binding site is not simply an acceptor molecule for a particular chemical class. The potencies of the serotonin receptor antagonists contrasts with those found for the *alpha*-blockers phentolamine, prazosine, and WB-4101, all of which were some 20-fold less active at the [^{3}H]THT site. Since it is known that trazodone can directly interact with both serotonin and $alpha_1$-adrenergic receptors (14, 16, 17), it was conceivable that the specifically bound isotope was attaching to either one or both of these sites. However, these experiments indicate that, of these two, the high-affinity [^{3}H]THT binding component is most likely the serotonin receptor. Moreover, since the serotonin receptor antagonists are some 600 times more active than the receptor agonists and mixed agonists-antagonists, it would seem that the [^{3}H]THT is behaving as an antagonist for this site (24).

It is also possible that [^{3}H]THT is attaching to some membrane component other than a neurotransmitter receptor. Such a site could represent a unique binding constituent for antidepressant drugs. However, this does not seem to be the case since even though the tricyclic antidepressants imipramine, desipramine, and doxepin, as well as the nontricyclic fluoxetine, displayed some activity as inhibitors of [^{3}H]THT binding, a variety of other antidepressants were inactive. Furthermore, the [^{3}H]THT binding site does not appear to be a nonspecific indole attachment site since some drugs possessing an indole moiety were also inactive.

Evidence to support an association between [^{3}H]THT and 5-HT$_1$ binding sites was provided by the finding that the regional distribution of [^{3}H]THT binding is similar to that reported for 5-HT$_1$ binding (25). For both sites the binding is highest in the cerebral cortex, corpus striatum, and hippocampus, and lowest in the cerebellum. Moreover, it is apparant that [^{3}H]THT binding is distinct from the 5-HT$_2$ recognition site since chronic treatment with imipramine induced a substantial reduction in 5-HT$_2$ receptor binding in frontal cortex but no significant change in [^{3}H]THT binding. Thus, the data from the pharmacological, lesion, and drug treatment studies suggest that [^{3}H]THT binding does not represent attachment to presynaptic serotonin sites, or recognition sites for 5-HT$_2$ or *alpha*-adrenergic receptors.

Studies have suggested that trazodone acts as both an agonist and antagonist with regard to serotonin-mediated behavior (16, 26). Since it has been found that a major metabolite of trazodone is *m*-chlorophenylpiperazine, a known serotonergic agonist (27, 28), it has been proposed that this substance mediates the agonist activity, whereas trazodone itself may be a serotonin receptor antagonist. The present data indicating that [^{3}H]THT may be an antagonist ligand for the 5-HT$_1$ binding site are consistent with these suggestions. Since both of the ligands currently used for labeling 5-HT$_1$ sites are agonists, with one of them (LSD) labeling both 5-HT$_1$ and 5-HT$_2$ sites (19), the discovery of an antagonist that may be specific for 5-HT$_1$ should aid in defining this binding component. Furthermore, a better definition of [^{3}H]THT binding could yield new insights into the mechanism of action of this antidepressant.

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