0022-3565/87/2421-0364$02.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 242, No. 1
Copyright © 1987 by The American Society for Pharmacology and Experimental Therapeutics
Printed in U.S.A.

Selective Labeling of Serotonin Uptake Sites in Rat Brain by [^{3}H]Citalopram Contrasted to Labeling of Multiple Sites by [^{3}H]Imipramine[1]

ROBERT J. D'AMATO, BRIAN L. LARGENT,[2] ADELE M. SNOWMAN and SOLOMON H. SNYDER

Departments of Neuroscience, Pharmacology and Molecular Sciences, Psychiatry and Behavioral Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland

Accepted for publication March 16, 1987

ABSTRACT

Citalopram is a potent and selective inhibitor of neuronal serotonin uptake. In rat brain membranes [^{3}H]citalopram demonstrates saturable and reversible binding with a K_D of 0.8 nM and a maximal number of binding sites (B_{max}) of 570 fmol/mg of protein. The drug specificity for [^{3}H]citalopram binding and synaptosomal serotonin uptake are closely correlated. Inhibition of [^{3}H]citalopram binding by both serotonin and imipramine is consistent with a competitive interaction in both equilibrium and kinetic analyses. The autoradiographic pattern of [^{3}H]citalopram binding sites closely resembles the distribution of serotonin. By contrast, detailed equilibrium-saturation analysis of [^{3}H]imipramine binding reveals two binding components, *i.e.*, high affinity (K_D = 9 nM, B_{max} = 420 fmol/mg of protein) and low affinity (K_D = 553 nM, B_{max} = 8560 fmol/mg of protein) sites. Specific [^{3}H] imipramine binding, defined as the binding inhibited by 100 μM desipramine, is displaced only partially by serotonin. Various studies reveal that the serotonin-sensitive portion of binding corresponds to the high affinity sites of [^{3}H]imipramine binding whereas the serotonin-insensitive binding corresponds to the low affinity sites. Lesioning of serotonin neurons with *p*-chloroamphetamine causes a large decrease in [^{3}H]citalopram and serotonin-sensitive [^{3}H]imipramine binding with only a small effect on serotonin-insensitive [^{3}H]imipramine binding. The dissociation rate of [^{3}H]imipramine or [^{3}H]citalopram is not altered by citalopram, imipramine or serotonin up to concentrations of 10 μM. The regional distribution of serotonin sensitive [^{3}H]imipramine high affinity binding sites closely resembles that of [^{3}H] citalopram binding. Thus, [^{3}H]citalopram and [^{3}H]imipramine appear to label common sites on the serotonin uptake complex. However, [^{3}H]imipramine also binds to a lower affinity site which is insensitive to serotonin and does not appear to be located primarily on serotonin neurons.

Attempts to label sites associated with the uptake of serotonin in platelets and neuronal membranes have used a variety of ligands including [^{3}H]imipramine (Talvenheimo *et al.*, 1979; Raisman *et al.*, 1979; Rehavi *et al.*, 1980; Langer *et al.*, 1981), [^{3}H]indalpine (Savaki *et al.*, 1985) and [^{3}H]paroxetine (Mellerup *et al.*, 1983; Habert *et al.*, 1985), with the majority of studies using [^{3}H]imipramine. Evidence that [^{3}H]imipramine does not bind in a single bimolecular fashion to the serotonin recognition site on the uptake complex includes shallow competition curves for serotonin against [^{3}H]imipramine binding to rat cortex (Sette *et al.*, 1983) and the slowing of [^{3}H]imipramine dissociation by serotonin (Wennogle and Meyerson, 1985; Severson *et al.*, 1986; Kim and Reith, 1986). Some studies suggest that [^{3}H] imipramine binds to more than one population of sites in brain membranes (Mocchetti *et al.*, 1982; Sette *et al.*, 1983; Whitaker *et al.*, 1983; Wood *et al.*, 1983, 1986; Hrdina, 1984; Conway and Brunswick, 1985; Severson *et al.*, 1986; Marcusson *et al.*, 1986).

Because citalopram is a very potent and selective inhibitor of serotonin uptake *in vitro* (Hyttel, 1982), we explored the use of [^{3}H]citalopram as a radioligand to label serotonin uptake sites. In the present study we present biochemical and autoradiographic evidence that [^{3}H]citalopram selectively labels serotonin uptake sites. By contrast, [^{3}H]imipramine binds to discrete high and low affinity sites with only the high affinity binding relating to serotonin uptake sites.

Methods

Binding studies. In membrane-homogenate binding studies, fresh whole brains (male Sprague-Dawley rats, 150–250 g), minus cerebella, were homogenized in 25 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4 at 25°C) containing 120 mM NaCl and 5 mM KCl (incubation buffer) and centrifuged at 45,000 × *g* for 10 min at 4°C. Pellets were suspended in fresh buffer and recentrifuged. This procedure was re-

Received for publication August 29, 1986.

[1] This work was supported by Grants DA-00266, MH-18501 and NS-16375 from Pfizer Pharmaceuticals, U.S. Public Health Service, RSA Award DA-00074 to S. H. S. and Training Grant GM-07309 to R. J. D.

[2] Present address: Department of Molecular Biology and Genetics, Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205.

ABBREVIATIONS: PCA, *p*-chloroamphetamine; B_{max}, maximal number of binding sites.

peated once more before membranes were finally suspended in an appropriate volume of incubation buffer for use in binding assays. This preparation typically resulted in a tissue homogenate containing approximately 6% of protein by original wet weight with the Pierce BCA protein assay reagent (Pierce Chemical Company, Rockford, IL).

In a final assay volume of 0.5 ml, 0.7 nM [^{3}H]citalopram (specific activity, 55 Ci/mmol) was incubated in the presence of various concentrations of unlabeled drug with the equivalent of 3.0 mg (original wet weight; approximately 180 μg of protein) of tissue for 60 min at room temperature. All experiments were performed in the linear tissue concentration range for binding and with an incubation time appropriate for the attainment of equilibrium. Nonspecific binding was assessed in the presence of 0.5 μM paroxetine.

Binding of [^{3}H]imipramine (specific activity, 66 Ci/mmol) was performed on rat brain membranes, with cerebella removed (tissue preparation modified as described by Raisman *et al.*, 1980), with the equivalent of 8 mg (original wet weight; approximately 480 μg of protein) of tissue in a final assay volume of 0.5 ml incubated for 60 min at 0°C in the presence of 1 nM [^{3}H]imipramine and varying concentrations of unlabeled drug. Nonspecific binding levels were estimated by inclusion of 100 μM desipramine.

Incubations for both radioligands were terminated by the addition of 4 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4 at 25°C with 120 mM NaCl-5 mM KCl; wash buffer) and membranes were collected by filtration under vacuum onto glass fiber filters (Schleicher and Schuell No. 32; pretreated with 0.5% polyethylenimine). Filters were washed with two consecutive 5-ml aliquots of wash buffer. The total time taken for the filtration/washing procedure was less than 10 sec. Radioactivity remaining on the filters was measured by liquid scintillation spectrometry at 60% efficiency. Equilibrium-saturation and drug competition binding data were analyzed with the iterative curve fitting computer programs EBDA (McPherson, 1983) and LIGAND (Munson and Rodbard, 1980). For dissociation experiments with either radioligand, dissociation was initiated by "infinite dilution" with 80-fold excess buffer (±drug) and, at various time points, the samples were filtered as stated above and the remaining radioactivity measured.

For studies examining lesions of the serotonergic neuronal systems, rats were injected daily with PCA (10 mg/kg i.p.) in saline for 3 consecutive days and were sacrificed 1 week after the last injection for preparation of brain tissues. The extent of lesioning of serotonergic neurons was estimated by high-performance liquid chromatography-electrochemical detection analysis of the tissue content of serotonin and its metabolites (Zaczek and Coyle, 1982).

Autoradiographic studies. Male Sprague-Dawley rats (150–250 g) were anesthetized with sodium pentobarbital and perfused *via* the left cardiac ventricle with 200 ml of 50 mM sodium phosphate (pH 7.5) containing 100 mM NaCl, followed by 200 ml of 50 mM sodium phosphate (pH 7.5) containing 0.3 M sucrose. After decapitation, brains were removed and mounted in a 1:1 mixture of bovine brain paste and Tissue-tek (Miles Laboratories, Naperville, IL) in plastic embedding molds, frozen rapidly at −40°C and then stored at −20°C. Tissues were mounted on microtome chucks and 10-μm sections were cut at −18°C and thaw-mounted on gelatin/chrom alum coated slides.

Before use the tissue sections were warmed to room temperature and preincubated in Tris-HCl buffer (pH 7.4 at 25°C) containing 120 mM NaCl and 5 mM KCl for 15 min at room temperature. In routine experiments slides were transferred from the preincubation buffer to 50-ml Coplin jars containing 1 nM [^{3}H]citalopram in 50 mM Tris-HCl buffer (pH 7.4 at 25°C) containing 120 mM NaCl and 5 mM KCl and incubated for 60 min at room temperature. Nonspecific binding was estimated by inclusion of 1 μM paroxetine in the incubation and was less than 10% of total binding in every instance. After the incubation, slides were dipped (1–2 sec) and then washed at 0°C (2 × 10 min) in incubation buffer, rinsed in distilled water and dried under a stream of cold dry air (30 sec) and then on a slide warmer (30°C; 30 min). Sections were then apposed to tritium-sensitive Ultrofilm (LKB, Gaithersburg, MD) at 4°C for 4 weeks to generate autoradiograms or removed with glass fiber filter discs to measure their radioactivity content by scintillation spectrometry.

A potential complication in the interpretation of *in vitro* autoradiography using tritiated ligands is the nonuniform quenching of β-emissions by gray and white matter regions. Rainbow *et al.* (1984) demonstrated a 30% reduction in apparent [^{3}H]ligand binding in pure white matter regions compared with gray matter. This differential quenching is a potential source of error when comparing absolute values of binding to gray matter with those for white matter. However, in this qualitative study, binding to white matter is sufficiently low that, even when allowing for the 30% differential, nonuniform quenching by white matter does not significantly change the relative levels of binding in gray matter to that in white matter.

Materials. [^{3}H]Citalopram and [^{3}H]imipramine (specific activity, 55 and 66 Ci/mmol, respectively) were supplied by NEN/Dupont Corporation (Boston, MA). Unlabeled drugs were obtained as follows: citalopram (HBr) and doxepin (HCl), Pfizer Pharmaceuticals (New York, NY); paroxetine (HCl), Ferrosan Pharmaceuticals (Copenhagen, Denmark); nortriptyline (HCl) and fluoxetine (HCl), Eli Lilly Pharmaceuticals (Indianapolis, IN); desipramine (HCl), USV Pharmaceuticals (Tuckahoe, NY); serotonin (creatinine sulfate) and PCA (HCl), Sigma Chemical Co. (St. Louis, MO); imipramine (HCl), Ciba-Geigy Pharmaceuticals (Summit, NJ); mazindol (HCl), Sandoz Pharmaceuticals (Hanover, NJ); desipramine (HCl), Merrell Pharmaceuticals (Cincinnati, OH); and nomifensine (maleate), Hoechst-Roussel Pharmaceuticals (Somerville, NJ). All other reagents were obtained from commercial sources.

Results

Equilibrium and kinetic properties of [^{3}H]citalopram binding. [^{3}H]Citalopram binds with high affinity to rat whole brain membranes in a saturable and reversible fashion. In typical experiments at room temperature using 0.7 nM [^{3}H] citalopram, total binding is about 3800 cpm, whereas nonspecific binding measured in the presence of 0.5 μM paroxetine is 400 cpm. Scatchard analysis of equilibrium-saturation binding experiments indicates a single population of binding sites with an apparent equilibrium-dissociation constant (K_D) of 0.84 ± 0.15 nM and a B_{max} of 570 ± 24 fmol/mg of protein (n = 4) (fig. 1).

[^{3}H]Citalopram binding is linear with tissue concentration from at least 0.005 to 0.5 mg of protein per 0.5 ml assay. Similar levels of equilibrium binding are apparent at incubation temperatures of 0°, 23° and 37°C. However, at 0°C at least a 3-hr incubation is required to attain equilibrium levels of binding. Accordingly, routine incubations are conducted at room temperature. By contrast, [^{3}H]imipramine binding assays must be conducted at 0°C as the ligand dissociates rapidly at higher temperatures, precluding reproducible assays (see below). [^{3}H] Citalopram binding is dependent upon the concentration of sodium cations. At room temperature, half-maximal binding occurs at 30 mM sodium and maximal binding is apparent at 100 mM sodium.

Both the association and dissociation rates of [^{3}H]citalopram are relatively slow (fig. 2). At room temperature half-maximal binding is attained after approximately 20 min of incubation, whereas binding plateaus at approximately 1 hr. The rate constant for association, as calculated by the second order integrated rate equation is 3.35 × 10^7 M/min. Dissociation experiments were conducted by infinite dilution of the radioligand with either buffer (80 volumes) or 100 μM unlabeled citalopram. Essentially the same results are obtained with both types of dissociation paradigms, indicating the absence of cooperative interactions. At room temperature, the dissociation

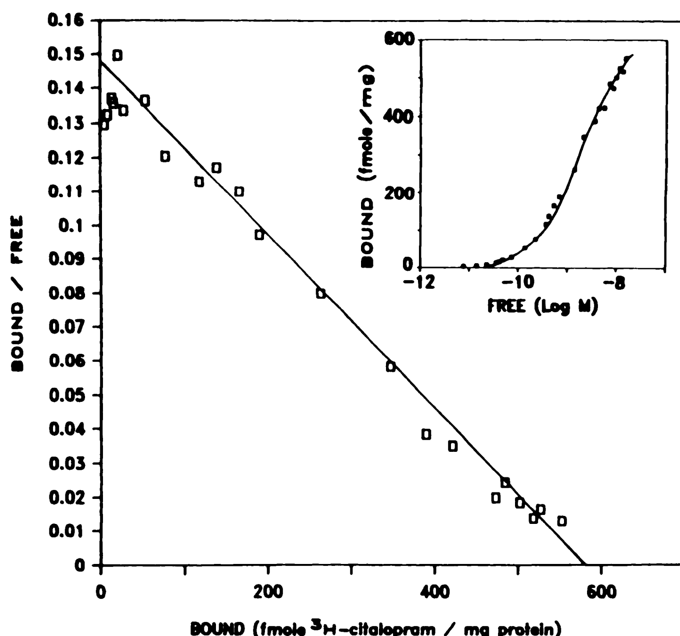

Fig. 1. Equilibrium-saturation binding of [³H]citalopram to membranes from whole rat brain, minus cerebellum. Membranes were incubated with various concentrations of [³H]citalopram (0.01–15 nM) for 60 min at room temperature. Nonspecific binding was estimated in the presence of 0.5 μM paroxetine. Presented results are from a typical experiment and values are the average of duplicate determinations repeated twice. The fit of this plot was derived from linear regression analysis. Analysis of this particular experiment resulted in a K_D of 0.95 nM and a B_{max} of 586 fmol/mg of protein. Insert: a plot of bound (femtomoles per milligram of protein) *vs.* free (log molar) [³H]citalopram.

of [³H]citalopram follows a monoexponential process with a half-life for dissociation of about 17 min. The rate constant for dissociation, derived from the plot of ln ($Bound_t/Bound_{Equil}$) *vs.* time (minutes), is 4.04×10^{-2}/min. The calculated equilibrium-dissociation constant determined kinetically from the ratio of the rate constant for dissociation to the rate constant for association is 1.2 nM, closely similar to the K_D value determined in equilibrium-saturation experiments.

Relationship of drug effects on [³H]citalopram binding and serotonin uptake. The relative potencies of drugs in competing for [³H]citalopram binding closely parallel their inhibitory effect upon serotonin uptake (table 1; fig. 3). Of the drugs examined, paroxetine is the most potent in inhibiting both [³H]citalopram binding and serotonin uptake whereas citalopram is the next most potent. All of the drugs examined, including tricyclic antidepressants and serotonin, inhibit [³H] citalopram binding with a pseudo-Hill coefficient of about 1.0, consistent with a competitive interaction between these drugs and [³H]citalopram binding sites. The apparently competitive nature of imipramine and serotonin inhibition of [³H]citalopram binding is emphasized by saturation analysis of [³H] citalopram binding in the presence of imipramine and serotonin. Thus, these drugs increase the apparent K_D of [³H] citalopram with no effect on its B_{max} for binding (fig. 4), an observation consistent with competitive interactions at the binding site.

Autoradiographic localization of [³H]citalopram binding. To assess further the relationship of [³H]citalopram binding to serotonin neuronal systems, we evaluated the autoradiographic localization of [³H]citalopram binding to rat brain sections (fig. 5). In preliminary experiments we examined the drug specificity of [³H]citalopram binding to 10 μm brain sections that are used for autoradiography. The affinity of [³H] citalopram itself and the K_i values in brain slices of serotonin (0.5–0.8 μM), paroxetine (0.2–0.4 nM) and imipramine (10–20 nM) are the same as in brain homogenates.

[³H]Citalopram binding sites are localized heterogeneously throughout the brain, matching the distribution of axons and terminals of serotonergic neurons. For example, the most intense densities of [³H]citalopram binding sites are localized discretely to the serotonergic raphe nuclei in the brainstem. Other areas known to be highly enriched in serotonergic nerve terminals, which also contain discrete high concentrations of [³H]citalopram associated grains, include the substantia nigra, locus ceruleus, superior colliculus, periaqueductal gray, interpeduncular nucleus, medial septum and ventral caudate. PCA treatment, producing a marked depletion of serotonin levels, destroys all [³H]citalopram binding in areas of serotonergic neuronal terminals (fig. 5). Interestingly, the medial forebrain bundle (fig. 5J) and serotonergic cell bodies (not shown) resist total depletion of [³H]citalopram binding sites.

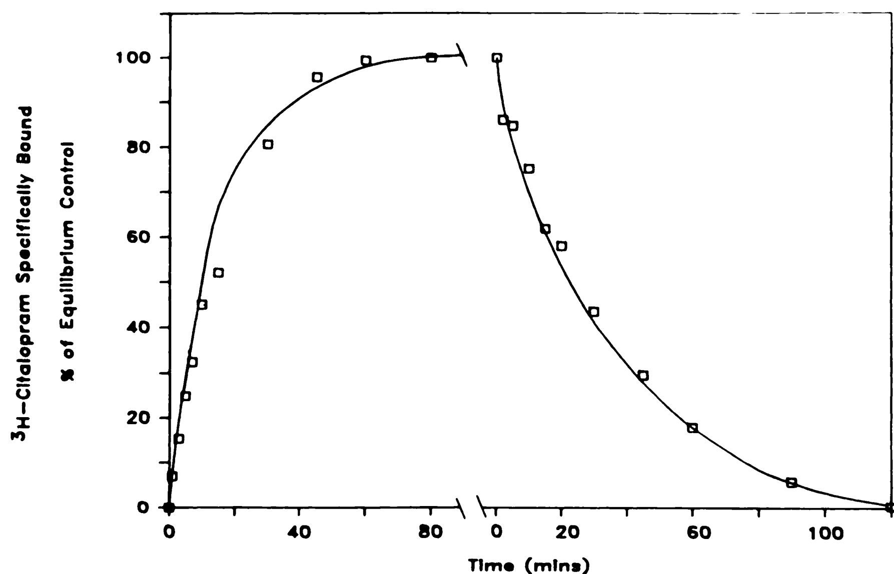

Fig. 2. Association and dissociation of [³H]citalopram at room temperature. Rat brain membranes were incubated with 0.7 nM citalopram for various lengths of time until equilibrium was established (60 min). Dissociation was initiated by 80 × dilution with incubation buffer and assay samples were filtered at various time points. Values presented are percentages of control levels observed at equilibrium and are the mean of two determinations done in duplicate. The association and dissociation rate constants are, as indicated in the text, 3.35 $\times 10^7$ M/min and 4.04×10^{-2}/min, respectively.

TABLE 1

Drug effects on [³H]citalopram binding and synaptosomal uptake of serotonin

K_i values (nanomolar) were determined from the data using computer-assisted iterative curve fitting with the EBDA program (McPherson, 1983) and are the mean of four determinations, done in duplicate, whose S.E.M. are less than 20%. Rat brain membranes were incubated with 0.7 nM [³H]citalopram for 60 min at room temperature in the presence of 20 to 22 concentrations of unlabeled drug in duplicate. Nonspecific binding was determined in the presence of 0.5 μM paroxetine.

Drug	[³H]Citalopram Binding K_i	Hill Slope for [³H]Citalopram Binding	Serotonin Uptake IC_{50}[a]
	nM		nM
Paroxetine	0.14	1.10	0.3
Citalopram	0.7	1.00	1.8
Chlorimipramine	1.0	1.03	1.5
Fluoxetine	5.0	1.01	6.9
Imipramine	12.0	.97	35.0
Mazindol	50.0	1.06	30.0
Doxepin	115.0	1.03	280.0
Desipramine	147.0	1.10	210.0
Serotonin	520.0	1.05	150.0
Nomifensine	990.0	1.08	830.0

[a] Included for comparison are IC_{50} (nanomolar) values for inhibition of synaptosomal uptake of [³H]serotonin taken from Hyttel (1982) or Wood *et al.* (1986). Correlating the log values of K_i for drugs against [³H]citalopram binding *vs.* log IC_{50} values for drug inhibition of synaptosomal serotonin uptake reveals a correlation coefficient (r) = 0.97.

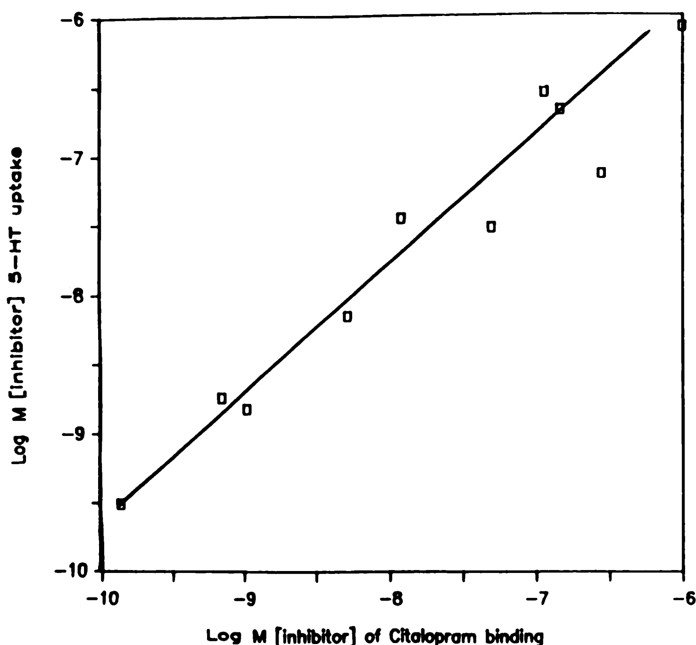

Fig. 3. Correlation of Log K_i values for [³H]citalopram binding sites *vs.* log inhibitory potency against [³H]serotonin (5-HT) synaptosomal uptake. Drug K_i values were taken from table 1. IC_{50} values for [³H]-5-HT uptake, also presented in table 1, were taken from Hyttel (1982) and Wood *et al.* (1986). The correlation coefficient (r) = 0.97 and the slope = 0.85 for the data presented.

Properties of [³H]imipramine binding. In confirmation of other reports (Langer *et al.*, 1981; Hrdina, 1984; Conway and Brunswick, 1985; Severson *et al.*, 1986), we find two components of [³H]imipramine binding (100 μM desipramine blank) (fig. 6). Analysis of equilibrium-saturation data by an iterative nonlinear curve-fitting computer program, LIGAND (Munson and Rodbard, 1980), indicates a high affinity component of binding with a K_D of 8.9 ± 1.7 nM and a B_{max} of 420 ± 41 fmol/mg of protein whereas the low affinity component exhibits a

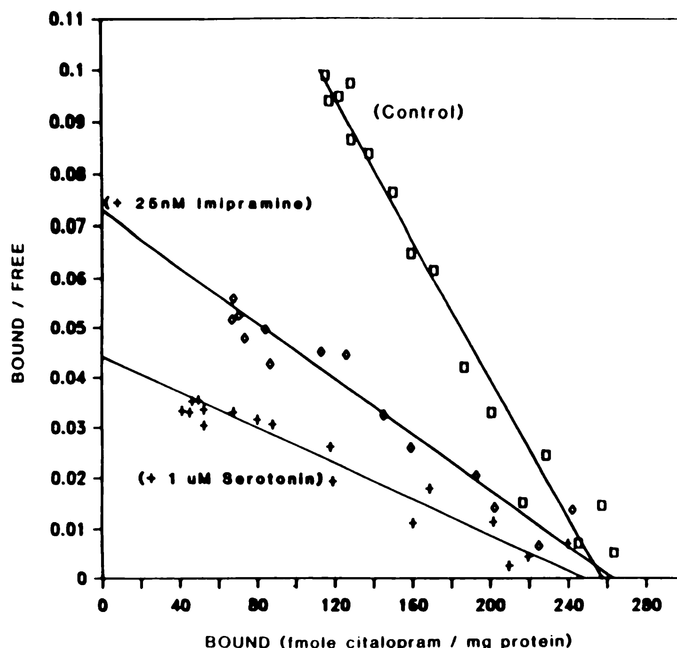

Fig. 4. Equilibrium-saturation binding of [³H]citalopram to rat whole brain membranes in the presence of 25 nM imipramine or 1 μM serotonin. Membranes were incubated with various concentrations of citalopram (0.01–1000 nM) and 0.7 nM [³H]citalopram at room temperature for 60 min. Results are from a typical experiment and are the average of duplicate determinations repeated twice within the same experiment. Fits for this plot were derived from linear regression analysis of the data.

K_D of 553 ± 76 nM and a B_{max} of 8560 ± 416 fmol/mg of protein (n = 4). Because of the high K_D of the low affinity site, the B_{max} value should be regarded as only approximate. In contrast to the monophasic inhibition of [³H]citalopram binding by serotonin, imipramine and various drugs, these compounds inhibit [³H]imipramine binding in a more complicated fashion, suggesting at least two distinct phases (fig. 7). In whole brain homogenates, serotonin inhibits maximally only about 45% of specific [³H]imipramine binding. Interestingly, B_{max} values are similar for both serotonin-sensitive [³H]imipramine binding, high affinity [³H]imipramine binding and [³H]citalopram binding. Although a discrete plateau is not apparent in the inhibition curve of [³H]imipramine by citalopram, curve fitting with the LIGAND program reveals two discrete components of binding with the high affinity component being similar in magnitude to the amount displaced by serotonin. Evidence that the serotonin-sensitive portion of [³H]imipramine binding represents the high affinity component comes from equilibrium-saturation analysis of [³H]imipramine binding in the presence of 100 μM serotonin. The high affinity binding is abolished by inclusion of 100 μM serotonin in the assay with Scatchard analysis of the serotonin-insensitive binding revealing one site with a K_D of 200 to 400 nM and a B_{max} of 3 to 5 pmol/mg of protein (n = 4) (data not shown).

To evaluate the relation of the two components of imipramine binding to serotonin uptake sites, we compared the drug specificity of high affinity (serotonin-sensitive) and low affinity (serotonin-insensitive) [³H]imipramine binding (table 2). The drug specificity for inhibition of serotonin-sensitive [³H]imipramine binding parallels the drug specificity of serotonin uptake. By contrast, there is no correlation between drug potencies at the serotonin-insensitive [³H]imipramine sites and inhibition of serotonin uptake. Drugs vary considerably in their

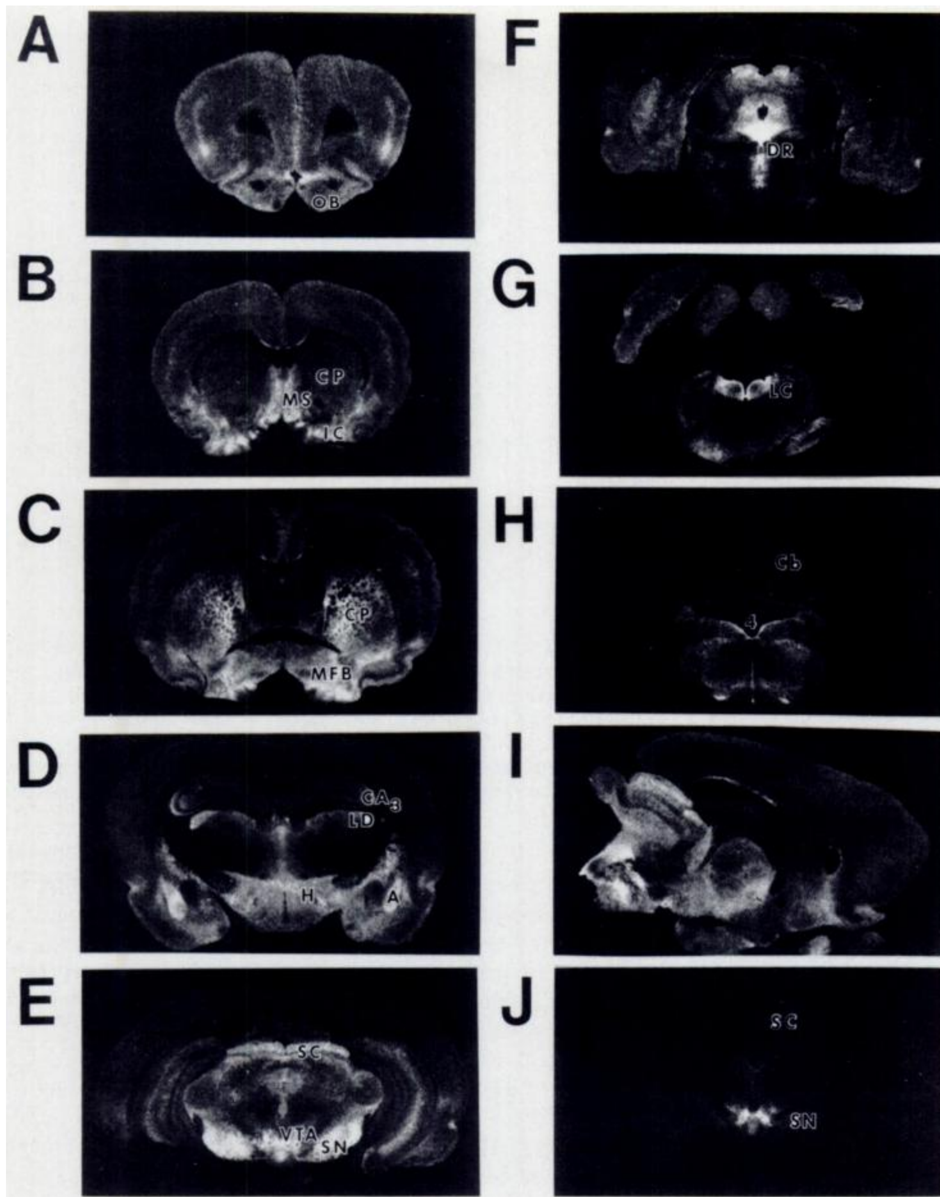

Fig. 5. Autoradiograms detailing the anatomical distribution of [^{3}H]citalopram binding in rat brain (A–I). Representative brain sections are presented in a rostral-caudal progression. Increased whiteness in photographs printed directly from Ultrofilm corresponds to higher levels of [^{3}H] citalopram binding-associated grains. As detailed under "Methods," 10-μM sections were incubated with 1 nM [^{3}H]citalopram for 60 min at room temperature, washed, dried and apposed to Ultrofilm for 2 months at 4°C. Nonspecific binding, measured in the presence of 1 μM paroxetine, is less than labeling seen over white matter in these plates. Structures were identified by reference to the atlas of Paxinos and Watson (1982). Section J (at the same level as E) is from an animal which was treated with 10 mg/kg of PCA (i.p.) for 3 consecutive days and sacrificed 1 week after the last injection. Note the loss of binding in the forebrain with the exception of some axonal pathways. Abbreviations are as follows: A, amygdala; CA3, field of Ammon's horn of hippocampus; Cb, cerebellum; CP, caudate/putamen; DR, dorsal raphe; H, hypothalamus; IC, Islands of Callaja; LD, lateral dorsal thalamic nucleus; LC, locus ceruleus; MFB, median forebrain bundle; OB, olfactory bulb; SC, superior colliculus; SN, substantia nigra; VTA, ventral tegmental area; 4, fourth ventricle.

relative effects on the serotonin-sensitive and serotonin-insensitive portions of [^{3}H]imipramine binding. Some drugs reduce the binding of both components similarly, whereas others are more effective at either the serotonin-sensitive or serotonin-insensitive portions of binding.

Experiments in which high (100 μM) concentrations of serotonin retard the dissociation of [^{3}H]imipramine from binding sites have suggested that serotonin influences imipramine binding allosterically (Wennogle and Meyerson, 1985; Severson *et al.*, 1986; Kim and Reith, 1986). We have compared the influence of serotonin upon [^{3}H]imipramine and [^{3}H]citalopram dissociation (fig. 8) and fail to find a significant influence of serotonin upon the dissociation rates of either [^{3}H]citalopram or [^{3}H]imipramine at concentrations of serotonin ranging from 10 nM to 10 μM. At 100 μM serotonin, we detect a modest slowing of [^{3}H]imipramine dissociation (data not shown) but have not been able to replicate this effect consistently.

Regional distribution of [^{3}H]citalopram and [^{3}H]imipramine binding and effects of serotonin neuronal lesions with PCA. The regional distribution of [^{3}H]citalopram binding in rat brain homogenates parallels the autoradiographic pattern of localizations, both indicating an association of binding sites with serotonergic neuronal systems (table 3). For instance, the highest levels of binding occur in the midbrain, which contains the raphe nuclei that are rich in serotonergic neurons and [^{3}H]serotonin uptake sites. Lowest levels of binding occur in the cerebellum, which contains a relatively sparse distribution of [^{3}H]citalopram associated autoradiographic grains.

The regional distribution of serotonin-sensitive (high affinity) [^{3}H]imipramine binding correlates well with that of [^{3}H] citalopram binding. By contrast, the serotonin-insensitive (low affinity) [^{3}H]imipramine binding does not correspond to [^{3}H] citalopram binding. For instance, the midbrain has 26 times more serotonin-sensitive [^{3}H]imipramine binding than the cerebellum, whereas the two regions have similar levels of serotonin-insensitive [^{3}H]imipramine binding. These results suggest that the low affinity binding is not linked physically to the

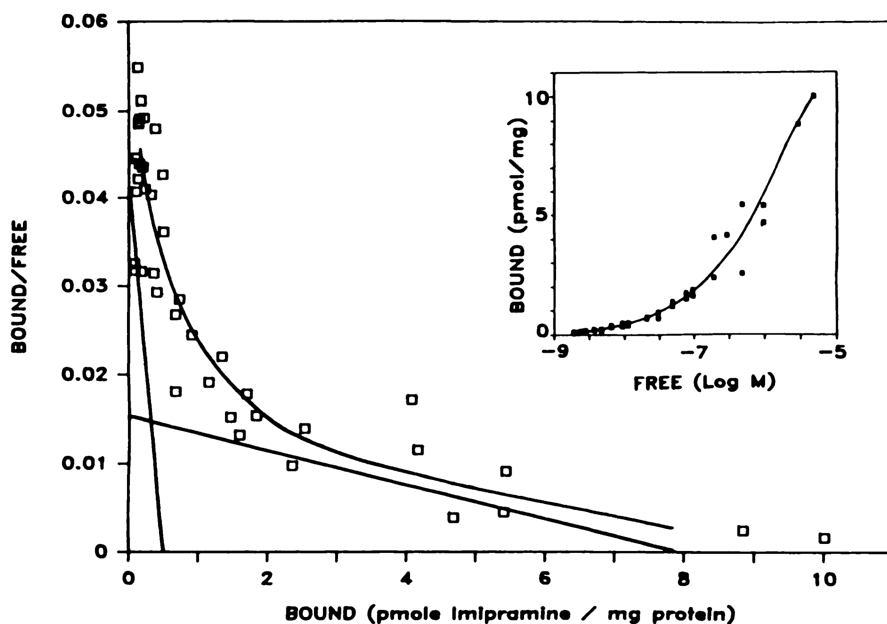

Fig. 6. Equilibrium-saturation binding of [^{3}H]imipramine to membranes from whole rat brain, minus cerebellum. Membranes were incubated with various concentrations of imipramine (1-7000 nM) and 1 nM [^{3}H]imipramine at 0°C for 60 min. Nonspecific binding was measured in the presence of 100 μM desipramine. Results are from a typical experiment and are the average of duplicate determinations repeated twice within the same experiment. Computer fit for this plot was performed by using iterative curve fitting with the LIGAND program (Munson and Rodbard, 1980). The straight lines represent the individual components of binding for this experiment with K_D = 11.7 nM and B_{max} = 0.48 pmol/mg of protein (high affinity) and K_D = 520 nM and B_{max} = 7.83 pmol/mg of protein (low affinity): Insert, a plot of the data as Bound (picomoles per milligram of protein) *vs.* free (log molar) imipramine.

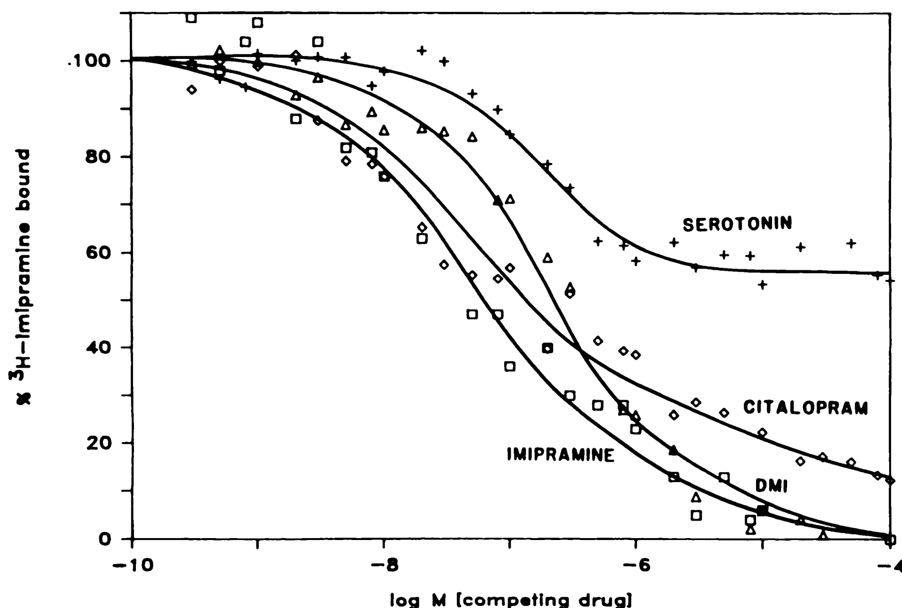

Fig. 7. Drug competition curves for [^{3}H]imipramine binding to whole rat brain membranes (minus cerebella). Membranes were incubated with 1 nM [^{3}H]imipramine with various concentrations of imipramine, serotonin, desipramine (DMI) or citalopram. Nonspecific binding was estimated with 100 μM DMI. Results are from a typical experiment and are the average of duplicate determinations repeated twice within the same experiment. The values represent percentage of control specific [^{3}H]imipramine bound. The computer fit for this plot was performed by using iterative curve fitting with the LIGAND program (Munson and Rodbard, 1980).

high affinity component of [^{3}H]imipramine binding and thus may be nonserotonergic. To evaluate the association of these various populations of binding sites with serotonergic neurons, we utilized PCA treatment, which destroys serotonergic terminals and depletes brain levels of serotonin markedly (table 4). Specific [^{3}H]citalopram binding is depleted 90% by PCA treatment, whether nonspecific binding levels are determined with serotonin (100 μM) or paroxetine (0.5 μM).

The extent of depletion of [^{3}H]imipramine binding by PCA treatment depends critically upon the way in which nonspecific binding levels are determined. Most studies in the literature of [^{3}H]imipramine binding use 100 μM desipramine to estimate nonspecific binding. When assayed in this fashion, PCA depletes only 54% of specific [^{3}H]imipramine binding. Utilizing 100 μM imipramine to assess nonspecific binding, a similar level of depletion is obtained. By contrast, the serotonin-sensitive component of [^{3}H]imipramine binding, *i.e.*, inhibited by 100 μM serotonin, is depleted 75% by PCA treatment, whereas the serotonin-insensitive component of [^{3}H]imipramine binding demonstrates only a 15% decrease. Thus, in the cerebral cortex it appears that about half of the total "desipramine-sensitive" [^{3}H]imipramine binding involves the serotonin uptake site. This proportion is even less in the cerebellum in which specific [^{3}H]imipramine binding (desipramine-sensitive) is decreased only 20% by PCA (data not shown). These values are consistent with the relative proportions of high and low affinity binding in these tissues with [^{3}H]imipramine at a concentration of 1 nM.

Discussion

A major finding of the present study is that [^{3}H]citalopram binds with considerable selectivity and high affinity to serotonin uptake sites. The loss of [^{3}H]citalopram binding upon destruction of serotonergic neurons, the autoradiographic localization of citalopram binding sites to areas containing sero-

TABLE 2

Drug effects on high and low affinity [^{3}H]imipramine binding and serotonin (5-HT) uptake

K_i values (nanomolar) were determined from the data using computer-assisted iterative curve fitting with the LIGAND program (Munson and Rodbard, 1980) and are the mean of three determinations done in duplicate. Rat whole brain membranes, minus cerebella, were incubated with 2 nM [^{3}H]imipramine and 20 to 22 concentrations of drug in duplicate for 60 min at 0°C. The K_D values for [^{3}H] imipramine were constrained to 9 nM for the high affinity site and 550 nM for the low affinity site, values obtained from the equilibrium-saturation data for [^{3}H] imipramine, when fitting drug competition data to two sites. The data fitted significantly better to a two-site model than a one-site model for every experiment ($P \leq .01$; F test).

	K_i		5-HT Uptake IC_{50}[a]
	High affinity site	Low affinity site	
	nM		*nM*
Paroxetine	2	6,509	0.3
Citalopram	7	4,250	1.8
Nortriptyline	115	3,100	590.0
Desipramine	146	5,406	210.0
5-HT	166	>500,000	150.0

[a] Included for comparison are IC_{50} (nanomolar) values for inhibition of synaptosomal uptake of [^{3}H]5-HT taken from Hyttel (1982) or Wood *et al.* (1986). Correlation coefficient of 5-HT uptake *vs.* log IC_{50} for the drug inhibition of high and low affinity sites are 0.98 and 0.22, respectively.

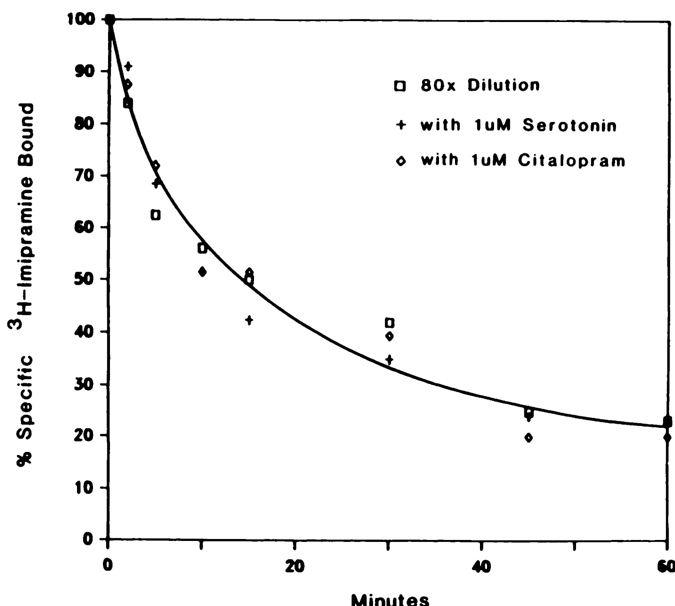

Fig. 8. Dissociation of [^{3}H]imipramine in the presence of serotonin or citalopram. Dissociation was initiated by infinite dilution with the addition of 80 volumes of buffer, alone or containing 1 μM serotonin or 1 μM citalopram. Nonspecific binding was determined at each time point by the inclusion of 100 μM desipramine to the initial incubation as in equilibrium saturation experiments.

tonergic axons and terminals, and the close correlation between drug potencies in inhibiting [^{3}H]citalopram binding and blocking of serotonin uptake all support the specificity of [^{3}H] citalopram interactions with the serotonin uptake system.

In contrast to the selective labeling of serotonergic neurons with [^{3}H]citalopram, [^{3}H]imipramine binding comprises two sites, only one of which involves serotonergic neurons. Evidence for this conclusion includes: 1) drug potencies at inhibiting synaptosomal serotonin uptake correlate with potencies at serotonin-sensitive but not serotonin-insensitive [^{3}H]imipramine binding; 2) the regional distribution of [^{3}H]citalopram binding parallels endogenous serotonin localizations and serotonin-sensitive but not serotonin-insensitive [^{3}H]imipramine binding; and 3) destruction of serotonin neuronal terminals by PCA depletes serotonin-sensitive but not serotonin-insensitive [^{3}H] imipramine binding. Very recently Marcusson *et al.* (1986) also showed that the serotonin-insensitive portion of [^{3}H]imipramine binding differs from serotonin uptake mechanisms in regional distribution, drug specificity and response to serotonin neuronal destruction. The extent to which [^{3}H]imipramine does not label serotonin neurons varies with different brain regions. In the cerebellum only about 20% of "specific" [^{3}H]imipramine binding involves serotonin uptake sites, as indicated by it's sensitivity to serotonin, whereas in the cerebral cortex this proportion is about 50%.

Autoradiographic labeling of serotonin neurons with [^{3}H] citalopram resembles that of [^{3}H]indalpine (Savaki *et al.*, 1985). However, [^{3}H]imipramine binding differs from that of [^{3}H] citalopram in that a maximum of 50% loss of [^{3}H]imipramine

TABLE 3

Brain regional distribution of [^{3}H]citalopram and [^{3}H]imipramine binding

Values for B_{max} of [^{3}H]citalopram binding were determined from equilibrium-saturation data using computer-assisted iterative curve fitting with the LIGAND program (Munson and Rodbard, 1980) and are the means of three independent determinations done in duplicate. [^{3}H]Imipramine data represent the mean of three independent determinations of serotonin displacement done in duplicate (up to 100 μM serotonin) of [^{3}H]imipramine binding while using 100 μM desipramine to estimate the level of nonspecific binding. Comparing B_{max} values for [^{3}H]citalopram binding to the amount of the serotonin-sensitive portion of [^{3}H]imipramine binding (S) reveals a correlation coefficient (r) = 0.92. The serotonin-insensitive component of [^{3}H]imipramine binding (I) is determined by subtracting the amount of serotonin-sensitive (S) binding from the total amount of specific [^{3}H]imipramine binding (defined as amount inhibited by 100 μM desipramine). The serotonin-insensitive portion of binding exhibits a poor correlation of drug potencies with [^{3}H]citalopram binding (correlation coeffiicent r = 0.56).

	[^{3}H]Citalopram B_{max}	[^{3}H]Imipramine		ratio S/I
		Serotonin-sensitive (S)	Serotonin-insensitive (I)	
	fmol/mg of protein	*Specific counts/mg of protein*		
Midbrain	1025 ± 75	4288 ± 423	1629 ± 256	2.6
Hypothalamus	963 ± 87	3980 ± 450	1912 ± 350	2.0
Thalamus	810 ± 31	3438 ± 389	1595 ± 292	2.2
Corpus striatum	652 ± 72	3343 ± 510	1841 ± 331	1.8
Medulla oblongata	610 ± 64	3093 ± 402	415 ± 135	7.5
Pons	605 ± 65	3170 ± 356	1050 ± 110	3.0
Hippocampus	559 ± 56	2639 ± 58	1218 ± 78	2.2
Cerebral cortex	480 ± 36	2279 ± 180	1082 ± 220	2.1
Spinal cord	476 ± 37	867 ± 89	788 ± 138	1.1
Cerebellum	130 ± 18	165 ± 23	1150 ± 46	0.1

TABLE 4

Effect of PCA treatment on levels of [^{3}H]citalopram and [^{3}H] imipramine binding in rat cerebral cortical membranes

Values presented are the mean (percentage) ± S.E.M. of depletion percentages for [^{3}H]citalopram or [^{3}H]imipramine binding in rat cerebral cortical membranes. Eight rats were treated with 10 mg/kg (i.p.) of PCA for 3 consecutive days and sacrificed 7 days after the last injection. Cerebral cortices from eight control and eight PCA-treated rats were assayed for [^{3}H]citalopram (0.7 nM) and [^{3}H]imipramine (1 nM) binding. Serotonin levels in cerebral cortex, measured by high-pressure liquid chromatography (Zaczek and Coyle, 1982), were depleted 87 ± 5% by PCA treatment, indicating the extent of lesioning. We have also observed a similar selective loss of [^{3}H]citalopram and serotonin-sensitive [^{3}H]imipramine binding in assays of other brain regions with varying proportions of serotonin-sensitive [^{3}H] imipramine binding (data not shown).

Blank	[^{3}H]Citalopram	[^{3}H]Imipramine
	% depletion	*% depletion*
Paroxetine (500 nM)	90 ± 5	
Serotonin (100 μM)	90 ± 5	74 ± 5
Desipramine (100 μM)		54 ± 4
Imipramine (100 μM)		56 ± 7
Serotonin-insensitive (Desipramine sensitive minus serotonin sensitive)		14 ± 10

binding is produced by serotonergic neurochemical lesioning (Dawson and Wamsley, 1983). Interestingly, the autoradiographic distribution of [^{3}H]imipramine in lesioned animals is still heterogenous and may reflect discrete localizations of the serotonin insensitive (low affinity) [^{3}H]imipramine binding sites. Severson *et al.* (1986) differentiated the binding of low concentrations of [^{3}H]imipramine into two components, one of which was sensitive to both sodium and serotonin whereas the other was insensitive. Lesions of serotonin-containing neurons with 5,7-dihydroxytryptamine reduced [^{3}H]imipramine binding to the serotonin- and sodium-sensitive component in a fashion similar to the lesions presented here with PCA. Although Severson *et al.* (1986) suggested that the serotonin-insensitive component has high affinity for imipramine, we suspect that it represents the same low affinity imipramine binding site that we have described here. Marcusson *et al.* (1985) noted that the high but not the low affinity component of [^{3}H]imipramine binding is sensitive to protease treatment whereas the low affinity component is resistant. Hrdina (1984) found that the drug specificity of the high affinity component of imipramine binding correlates with inhibition of serotonin uptake.

Other reports have suggested that the complex inhibition of [^{3}H]imipramine binding by serotonin and various drugs is explained by allosteric influences of these compounds upon the binding of [^{3}H]imipramine to a single site which involves the serotonin neuronal uptake mechanism (Sette *et al.*, 1983). The discrimination of [^{3}H]imipramine binding sites into two components with only one involving serotonergic neurons may account for the complex effects of drugs on [^{3}H]imipramine binding, as suggested also quite recently by Marcusson *et al.* (1986). The slowing of [^{3}H]imipramine dissociation by serotonin, at concentrations of 100 μM or greater, has also been proposed to reflect allosteric influences (Severson *et al.*, 1986; Kim and Reith, 1986). However, at 1 to 10 μM concentrations of serotonin we have not been able to detect such effects in brain membranes, even though these concentrations should saturate the serotonin uptake process. The most prominent influences of serotonin on imipramine dissociation have been reported in platelets, whereas in brain membranes dissociation is slowed only about 2-fold by very high concentrations of serotonin. It is unclear whether the influence of serotonin on [^{3}H]imipramine dissociation reflects an action at serotonin uptake sites.

Acknowledgments

The secretarial assistance of Rita Hollingsworth is very much appreciated. We thank Dr. Jay Baraban for critical discussions of various aspects of this work.

References

Conway, P. and Brunswick, D.: High and low affinity binding components for [^{3}H]imipramine in rat cerebral cortex. J. Neurochem. **45**: 206-209, 1985.

Dawson, T. M. and Wamsley, J. K.: Autoradiographic localization of [^{3}H] imipramine binding sites: Association with serotonergic neurons. Brain Res. Bull. **11**: 325-334, 1983.

Habert, E., Graham, D., Tahraoui, L., Claustre, Y. and Langer, S. Z.: Characterization of [^{3}H]paroxetine binding to rat cortical membranes. Eur. J. Pharmacol. **118**: 107-114, 1985.

Hrdina, P. D.: Differentiation of two components of specific [^{3}H]imipramine binding in rat brain. Eur. J. Pharmacol. **102**: 481-488, 1984.

Hyttel, J.: Citalopram; basic and clinical studies. Prog. Neuropharmacol. Biol. Psychiatry **6**: 275-336, 1982.

Kim, S. and Reith, M.: Effect of serotonin on the dissociation of high affinity binding of [^{3}H]imipramine in mouse cerebral cortex. Neurosci. Lett. **67**: 123-128, 1986.

Langer, S. F., Javoy-Agid, F., Raisman, R., Briley, M. and Agid, Y.: Distribution of specific high affinity binding sites for [^{3}H]imipramine in human brain. J. Neurochem. **37**: 267-271, 1981.

Marcusson, J. O., Backstrom, I. T. and Ross, S. B.: Single site model of the neuronal 5-hydroxytryptamine uptake and imipramine-binding site. Mol. Pharmacol. **30**: 121-128, 1986.

Marcusson, J. O., Fowler, C. J., Hall, H., Ross, S. B. and Winblad, B.: "Specific" binding of [^{3}H]imipramine to protease-sensitive and protease-resistant sites. J. Neurochem. **44**: 705-711, 1985.

McPherson, G. A.: A practical computer-based approach to the analysis of radioligand binding experiments. Computer Programs Biomed. **17**: 107-114, 1983.

Mellerup, E. T., Plenge, P. and Engelstoft, M.: High affinity binding of [^{3}H]paroxetine and [^{3}H]imipramine to human platelet membranes. Eur. J. Pharmacol. **96**: 303-309, 1983.

Mocchetti, I., Brunello, N. and Racagni, G.: Ontogenetic study of [^{3}H] imipramine binding sites and serotonin uptake system: Indication of possible interdependence. Eur. J. Pharmacol. **83**: 151-152, 1982.

Munson, P. J. and Rodbard, D.: LIGAND: A versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. **107**: 220-239, 1980.

Paxinos, G. and Watson, C.: The Rat Brain in Stereotaxic Coordinates, Academic Press, Sydney, 1982.

Rainbow, T. C., Biegon, A. and Berek, D. J.: Quantitative receptor autoradiography with tritium-labeled ligands: Comparison of biochemical and densitometric measurements. J. Neurosci. Sci. Methods **11**: 231-241, 1984.

Raisman, R., Briley, M. and Langer, S. Z.: High affinity ^{3}H-imipramine binding in rat cerebral cortex. Eur. J. Pharmacol. **54**: 307-308, 1979.

Rehavi, M., Paul, S. M. Skolnick, P. and Goodwin, F. K.: Demonstration of specific high affinity binding sites for [^{3}H]imipramine in human brain. Life Sci. **26**: 2273-2279, 1980.

Savaki, H., Malgouris, C., Benavides, J., Laplace, C., Uzan, A., Gueremy, C. and Le Fur, G.: Quantitative autoradiography of [^{3}H]indalpine binding sites in the rat brain. II. Regional distribution. J. Neurochem. **45**: 521-526, 1985.

Sette, M., Briley, M. S. and Langer, S. Z.: Complex inhibition of [^{3}H] imipramine binding by serotonin and nontricyclic serotonin uptake blockers. J. Neurochem. **40**: 622-628, 1983.

Severson, J. A., Woodward, J. J. and Wilcox, R. E.: Subdivision of mouse brain [^{3}H]imipramine binding based on ion dependence and serotonin sensitivity. J. Neurochem. **46**: 1743-1754, 1986.

Talvenheimo, J., Nelson, P. and Rudnick, G.: Mechanism of imipramine inhibition of platelet 5-hydroxytryptamine transport. J. Biol. Chem. **254**: 8606-8611, 1979.

Wennogle, L. and Meyerson, L.: Serotonin uptake inhibitors differentially modulate high affinity imipramine dissociation in human platelet membranes. Life Sci. **36**: 1541-1550, 1985.

Whitaker, P. M., Vint, C. K. and Morin, R.: [^{3}H]Imipramine labels sites on brain astroglial cells not related to serotonin uptake. J. Neurochem. **41**: 1319-1323, 1983.

Wood, M., Broadhurst, A. and Wyllie, M.: Examination of the relationship between the uptake system for 5HT and high affinity [^{3}H]imipramine binding site. Neuropharmacology **25**: 519-525, 1986.

Wood, P. L., Suranyi-Cadotte, B. E., Nair, N. P. V., LaFaille, F. and Schwartz, G.: Lack of association between [^{3}H]imipramine binding sites and uptake of serotonin in control, depressed and schizophrenic patients. Neuropharmacology **22**: 1211-1214, 1983.

Zaczek, R. and Coyle, J.: Rapid and simple method for measuring biogenic amines and metabolites in brain homogenates by HPLC-EC detection. J. Neural Transm. **53**: 1-5, 1982.

Send reprint requests to: Dr. Soloman H. Snyder, Departments of Neuroscience, Pharmacology and Molecular Sciences, Psychiatry and Behavioral Sciences, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205.