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Single-Site Model of the Neuronal 5-Hydroxytryptamine Uptake and Imipramine-Binding Site

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SUMMARY

Recently, a high affinity [^{3}H]imipramine-binding site of protein nature that appeared to be related to the 5-hydroxytryptamine (5-HT, serotonin) uptake mechanism was demonstrated. This binding site was only part of desipramine-displaceable [^{3}H]imipramine binding, which contained a significant amount of additional binding not related to 5-HT uptake. The present study further investigates the [^{3}H]imipramine-binding site of protein nature in the rat brain. Displacement by 5-HT and 6-methoxytetrahydro-β-carboline (6-MeO-THβC) revealed monophasic displacement patterns with 60% displaceable binding. This binding fraction was abolished by protease treatment of the brain tissue prior to binding assay. Saturation studies of [^{3}H]imipramine binding (1–30 nM) in rat cortex showed that the binding displaced by 30 μM 5-HT [B_{max} 322 ± 16 fmol/mg of protein, K_d 4.17 ± 1.07 nM (means ± SE)] was not different from the binding displaced by 1.0 μM norzimeldine (B_{max} 349 ± 15 fmol/mg of protein, K_d 4.47 ± 1.07 nM) or 30 μM 6-MeO-THβC (B_{max} 439 ± 28 fmol/mg of protein, K_d 5.49 ± 1.09 nM). When 100 μM desipramine was used in saturation studies, the binding was different from that displaced by 5-HT with B_{max} 608 ± 42 fmol/mg of protein and K_d 6.68 ± 1.09 nM. Both displacement and saturation studies in which two displacing agents were combined indicated that most of the binding competed by 5-HT (30 μM) and norzimeldine (1.0 μM) is identical. Similarly, the binding displaced by 5-HT or norzimeldine is subsumed within 6-MeO-THβC (30 μM)-displaceable binding. Lesion studies with *para*-chloroamphetamine, a selective toxin for 5-HT terminals, which resulted in a 83% reduction of [^{3}H] 5-HT uptake ([^{3}H]noradrenaline uptake unaffected), abolished cortical [^{3}H]imipramine binding displaced by 30 μM 5-HT or 1.0 μM norzimeldine. (>80% reduction). However, with 100 μM desipramine as displacer, 40% of the binding remained in lesioned animals. The [^{3}H]imipramine binding displaced by 30 μM 5-HT or 1.0 μM norzimeldine was sodium dependent, and an increase in NaCl concentration from 0 to 120 mM resulted in a 10-fold increase in affinity without effect on B_{max}, whereas no change in binding was observed with increasing concentrations of LiCl. The regional distribution of [^{3}H]imipramine binding displaced by 5-HT, norzimeldine, or 6-MeO-THβC correlated with [^{3}H] 5-HT uptake, with the highest binding capacity in the hypothalamus and the striatum (B_{max} 410–480 fmol/mg of protein), and the K_d values for the different regions remained constant (4–5 nM). The binding displaced by 100 μM desipramine showed 2-fold higher B_{max} values throughout the brain, the K_d values being 6–9 nM except for the hypothalamus with K_d 4 nM. Finally, the pharmacological interactions on the [^{3}H]imipramine-binding site of protein nature, discriminated by 30 μM 5-HT or 1.0 μM norzimeldine, was studied. Both norzimeldine and 5-HT were found to inhibit the binding in a competitive manner. Similarly, imipramine inhibited competitively neuronal [^{3}H] 5-HT uptake, and a competitive interaction by norzimeldine on [^{3}H] 5-HT uptake has previously been reported. Based on these studies, it is suggested that the binding site for 5-HT uptake inhibitors causing inhibition of 5-HT uptake is identical to the substrate recognition site for neuronal 5-HT uptake and that the 5-HT uptake transporter can be selectively studied in brain tissue using [^{3}H]imipramine.

Binding sites for the tricyclic antidepressant imipramine have been demonstrated in brain tissues (1, 2). Several data indicate that [^{3}H]imipramine-binding sites are related to the 5-HT uptake mechanism. The density of these sites appears to correlate with the distribution of endogenous 5-HT (3), and significant correlations between the abilities of antidepressants to inhibit 5-HT uptake and to displace [^{3}H]imipramine binding have been found (4, 5). Although these findings point to an identity between the [^{3}H]imipramine-binding sites and the sites recognizing 5-HT for transport, some other observations indicate that these sites are not identical but interact allosterically with each other (6). Thus, the inhibition by 5-HT of the [^{3}H]imipramine binding was reported to be complex and noncompetitive. Furthermore, non-tricyclic 5-HT uptake inhibitors inhibited the [^{3}H]imipramine binding in a complex manner with Hill coefficients much lower than unity (6).

These complexities in the inhibition of [^{3}H]imipramine bind-

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine, serotonin; 6-MeO-THβC, 6-methoxytetrahydro-β-carboline, also referred to as endotryptoline or 5-methoxytryptoline; PCA, *para*-chloroamphetamine hydrochloride; NA, noradrenaline.

ing can be explained by the observed heterogeneity of the [^{3}H] imipramine-binding sites. High and low binding sites for [^{3}H] imipramine have been reported (7–9). Marcusson *et al.* (10) found that the non-tricyclic 5-HT uptake inhibitor, norzimeldine, discriminated a high affinity [^{3}H]imipramine-binding site of proteinaceous nature which correlated with the regional distribution of 5-HT uptake. However, when desipramine was used to define "specific" binding, a significant amount of additional low affinity, non-proteinaceous binding not related to 5-HT uptake was obtained (10). Since the model of the [^{3}H] imipramine-binding site and 5-HT uptake complex is based on studies in which desipramine has been used in the definition of "specific" binding, the model is now further investigated focusing on the [^{3}H]imipramine-binding sites of protein nature. This study presents a comparison between the pharmacological properties of [^{3}H]imipramine-binding sites of protein nature and desipramine-displaceable sites. In addition, the comparison involves the effect of Na^+ and Li^+ ions, the cellular location of the sites, and their regional distribution. The compound 6-Meo-THβC, which has been suggested as an endogenous ligand of the imipramine-binding site (11, 12), is also included in the study. In contrast to previous reports, it is concluded that only a fraction of [^{3}H]imipramine-binding sites, which is of protein nature and is discriminated by 5-HT and norzimeldine, can be regarded as real specific binding. Furthermore, studies of the pharmacological interactions suggest that the [^{3}H]imipramine-binding site described is the substrate recognition site for 5-HT uptake, thus forming a unitary model for 5-HT uptake and [^{3}H]imipramine-binding sites.

Experimental Procedures

Materials. [*N-methyl*-^{3}H]Imipramine hydrochloride (75.6–77.4 Ci/mmol) and (−)-[7-^{3}H]NA (14.8 Ci/mmol) were obtained from New England Nuclear (Boston, MA). [G-^{3}H]5-HT creatinine sulfate was obtained from Amersham International plc (Amersham, U.K.). Nonradioactive 5-HT creatinine sulfate was obtained from Merck (Darmstadt, West Germany). Norzimeldine dihydrochloride hydrate was obtained from Astra Läkemedel AB, Södertälje, Sweden, and 6-MeO-THβC was synthesized by Dr. L. Florvall, Astra Läkemedel AB, Södertälje, Sweden. Desipramine hydrochloride and imipramine hydrochloride were donated by CIBA-Geigy AG, Basel, Switzerland. PCA was purchased from Sigma Chemical Co., St. Louis, MO. All other reagents were standard laboratory reagents of analytical grade wherever possible.

[^{3}H] Imipramine binding. Male Sprague-Dawley rats (180–220 g) were decapitated, brains were dissected, and specific regions were immediately frozen on dry ice and then stored at −70° until use. The brain tissues were homogenized in 15 ml of ice-cold buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, pH 7.40) using a Kinematica Polytron homogenizer (Lüzern, Switzerland), setting 4, 10 sec. After centrifugation (48,000 × *g*, 10 min, 4°), the pellet was homogenized in the Tris-HCl buffer and, following centrifugation (48,000 × *g*, 10 min), resuspended in the buffer to a final tissue concentration of 500–800 μg of protein/ml in the binding assay. The homogenates were incubated for 60 min at 0° with [^{3}H]imipramine in the absence or presence of the displacing agent under test in a total volume of 450 μl. After addition of 6 ml of ice-cold buffer, the homogenates were rapidly filtered through Whatman GF/C filters, followed by three 6-ml rinses with cold buffer, using a 24-channel cell harvester (Brandel, Gaithersburg, MD). The radioactivity trapped by the filters was determined by liquid scintillation spectroscopy. The binding was found to be linear with the tissue concentration in the range tested (200–1500 μg of protein/ml).

Synaptosomal uptake of [^{3}H]5-HT and [^{3}H]NA. Crude synaptosomal preparations were made by homogenizing the tissues in 10 volumes of ice-cold 0.32 M sucrose with all glass Potter-Elvehjem homogenizers. The homogenates were centrifuged at 800 × *g* at 2° for 10 min. The supernatants were centrifuged at 18,000 × *g* for 10 min and the pellets (P_2 fraction) were rehomogenized to the original volume in 0.32 M sucrose. The incubation of the preparations with [^{3}H]5-HT and [^{3}H]NA was performed in a Micronic R PPN Storage block, 96 m (Flow Laboratories, Irvine, Scotland) with 8 × 12 wells. One row of wells was used for uninhibited uptake and the second row for inhibited (nonspecific) uptake. Six concentrations, between 20 and 200 nM, of the labeled amines in duplicate were used. Fifty μl of the synaptosomal preparation corresponding to 5 mg wet weight of the tissue and 425 μl of the Krebs-Henseleit buffer, pH 7.4, containing 5.6 mM glucose, 1.1 mM ascorbic acid, 0.13 mM disodium edetate, and 50 μM pargyline, were added to the wells in the first row and the same buffer containing 1.2 μM citalopram (5-HT uptake) or 10 μM maprotiline (NA uptake) to the wells in the second row. The solutions were mixed by vortexing the block for 10 sec. After 10 min of preincubation at 37° in a water bath, 25 μl of the [^{3}H]5-HT or [^{3}H]NA solutions were added to the two rows with a Titertec multichannel pipette, type 12 channel (Flow Laboratories). The reaction was immediately started by vortexing the block for 10 sec, and the incubation continued for 2 min at 37°. The uptake reaction was stopped by filtration and washing for 15 sec with ice-cold 150 mM NaCl through Whatman GF/B glass filter paper in a 24-channel Brandel Cell Harvester using the standard cell harvesting probe. The radioactivity trapped by the filters was determined by liquid scintillation spectroscopy. The active uptake was calculated as the difference between the accumulation of the labeled amine in the synaptosomes in the absence and presence of the uptake inhibitor (citalopram or maprotiline). The uptake processes of [^{3}H]5-HT and [^{3}H]NA were linear with respect to incubation time, and the uptake inhibitors approached 100% inhibition at all concentrations of the labeled amines examined. The uptake was calculated in pmol/g of wet weight tissue/min. The kinetics constants (V_{max} and K_m) were calculated by regression analysis from Eadie-Hofstee plots.

Protease sensitivity experiment. In one set of experiments, the homogenate obtained after the first centrifugation was incubated for 75 min at 37° in the absence or presence of a protease (Protease P5380, Sigma; approximately 0.5% of wet weight brain tissue content). After the protease incubation the homogenates were centrifuged again at 48,000 × *g* for 10 min, resuspended, and then used in the binding assay.

Na^+ dependency study. Using the same procedure for [^{3}H]imipramine binding as described above, the buffer in all preparation steps was varied with respect to NaCl concentration. In one set of experiments the NaCl concentrations were 0, 50, 120, 250, 500 mM. In another set of experiments, NaCl was replaced by LiCl of the same concentrations (0–500 mM).

Lesion experiments. In the lesion studies, male Sprague-Dawley rats (180–220 g) were injected intraperitoneally twice (24-hrs interval) with PCA (10 mg/kg). Control animals were treated the same way but with physiological saline instead of PCA. Lesioned and control animals were decapitated 7 days after final injection, whereafter the brain tissues were either assayed for 5-HT and NA uptake or stored at −70° until binding experiments were performed.

Regional distribution study. For the regional distribution study, rats were divided into two groups, one for the [^{3}H]imipramine binding experiments and one for the 5-HT and NA uptake assays. The dissected regions were either frozen immediately for subsequent binding studies or homogenized in 10 volumes of ice-cold 0.32 M sucrose in Potter-Elvehjelm all-glass homogenizers. The homogenates were centrifuged at 800 × *g* for 10 min and the cell-free supernatants were used for the uptake experiments.

Kinetics of the inhibition of [^{3}H]5-HT uptake by imipramine. Rat cerebral cortices were homogenized and centrifuged as described above, but the P_2 fraction was washed once (rehomogenized and centrifuged) with 0.32 M sucrose. The incubation was performed according to the standard procedure described with the exception that 25 μl of imipramine giving final concentrations of 25, 50, or 100 nM were added to the second row and 25 μl of buffer solution to the first row, and 400

μl of buffer solutions were added to all wells. Within 5 min the same experiments were performed, but with buffer (400 μl) containing citalopram (1.2 μM final concentration) added to all wells.

Data analysis and statistics. Values for maximum binding (B_{max} and apparent affinity (K_d) were determined by Scatchard analysis where best-fitting lines to the data points were calculated by least squares linear regression analysis. Apparent K_i values for drug inhibition in saturation studies were calculated according to the formula for competitive inhibition (13):

$$K_i = \frac{K_d \times [i]}{K_d - Kd_i}$$

where K_d is the apparent affinity in the absence and Kd_i is affinity in the presence of the inhibitor, i. The concentration of each drug giving half-maximal displacement (IC_{50}) was calculated by linear regression analysis of log-logit plots of the inhibition curves. Hill coefficients (n_H) were determined from Hill plots of saturation experiments. Data shown in figures are mean plots of the number of experiments indicated. The statistical analysis involved multiple comparisons and, therefore, if not otherwise stated, analysis of variance with *à postiori* testing of significance by Tukey's test (14) was performed. The criterion for significance was $p < 0.05$. The individual B_{max} and pK_d values from separate experiments were used in these analyses. When given as mean values, the B_{max} values represent arithmetic and the K_d values geometric values.

Results

Displacement studies. The displacement of [^{3}H]imipramine binding from rat cortical membranes by 5-HT revealed a monophasic displacement pattern with an IC_{50} of 0.5 μM. About 60% of the total binding was displaceable (Fig. 1A). A similar displacement pattern was found with 6-MeO-THβC: 60% displacement of total binding, IC_{50} 0.3 μM (Fig. 1D). The high affinity component of norzimeldine displacement represented 60% of total binding at 1.0 μM (IC_{50} 9 nM). To determine the relation between the [^{3}H]imipramine binding displaceable by

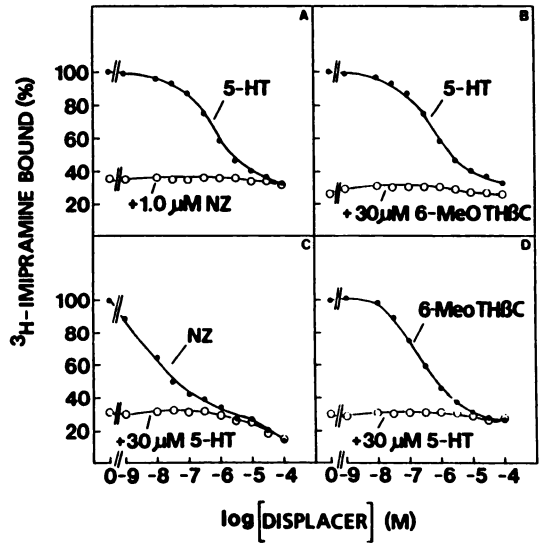

Fig. 1. Drug displacement of [^{3}H]imipramine (2.5 nM) binding of rat cortical membranes. Results are expressed as percentage of total binding in the absence of displacer. The data represent mean values of three experiments and the standard error of each data point was <10%. Increasing concentrations of (A) 5-HT separately (●) and in combination with 1.0 μM norzimeldine (*NZ*) (○); (B) 5-HT separately (●) and in combination with 30 μM 6-MeO-THβC (○); (C) norzimeldine separately (●) and in combination with 30 μM 5-HT (○); or (D) 6-MeO-THβC separately (●) and in combination with 30 μM 5-HT (○) were used.

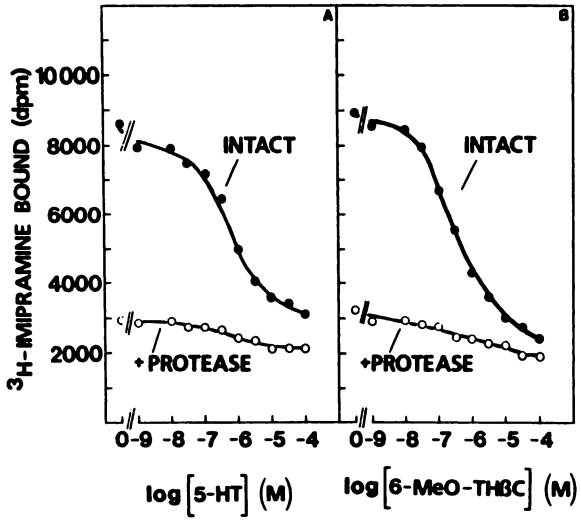

Fig. 2. Drug displacement of [^{3}H]imipramine (2.5 nM) binding to control and protease-treated rat cortical membranes. Samples were preincubated in the absence (●) or presence (○) of protease (0.5% of tissue wet weight) for 75 min before binding assay (see Experimental Procedures). Results are expressed as disintegrations per minute (dpm) and values are means of three experiments, the standard error of each data point being <10%. Increasing concentrations of (A) 5-HT and (B) 6-MeO-THβC were used.

5-HT, norzimeldine, and 6-MeO-THβC, respectively, the competition by 5-HT was performed in the presence of 1.0 μM norzimeldine and 30 μM 6-MeO-THβC, respectively (Fig. 1, A and B). In addition, the displacement by norzimeldine and 6-MeO-THβC was performed in the presence of 5-HT (Fig. 1, C and D). The addition of 1.0 μM norzimeldine did not augment the displacement of [^{3}H]imipramine by 5-HT (Fig. 1A). This indicates that the [^{3}H]imipramine-binding sites displaced by 5-HT are identical to the binding sites with high affinity (<1.0 μM) for norzimeldine. Similarly, when 30 μM 5-HT was added to the norzimeldine competition, only the high affinity component of the norzimeldine displacement (<1.0 μM) was eliminated (Fig. 1C). Similar findings were noted when 5-HT and 6-MeO-THβC were combined, even though a small additional displacement was obtained when 30 μM 6-MeO-THβC was added to the 5-HT competition (Fig. 1, B and D).

Protease experiments. It was previously shown that [^{3}H]imipramine-binding sites with high affinity (<1.0 μM) for norzimeldine are protease sensitive (see Ref. 10). The [^{3}H]imipramine binding displaceable by 5-HT and 6-MeO-THβC, respectively, was also found to be sensitive to protease treatment (Fig. 2).

Saturation studies. The nonspecific binding was defined by 5-HT (30 μM), norzimeldine (1.0 μM), 6-MeO-THβC (30 μM), or desipramine (100 μM) in saturation experiments with increasing concentrations of [^{3}H]imipramine. A comparison between each concentration of the saturation curves for 5-HT- and norzimeldine-displaceable binding revealed no significant differences (Fig. 3, A and B) and, consequently, no differences in either B_{max} or K_d were obtained (see legend to Fig. 3). The binding displaced by 30 μM 6-MeO-THβC had a tendency for higher K_d and B_{max} values, but this was reflected in a significant difference only at the highest [^{3}H]imipramine concentration used (Fig. 3C). When 100 μM desipramine was used to define nonspecific binding, a significant increase in B_{max} and K_d was

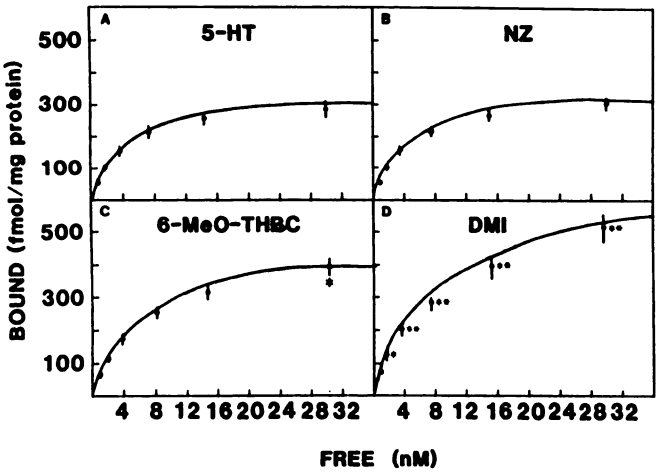

Fig. 3. Saturation curves for [^{3}H]imipramine binding to rat cortical membranes. Depicted is the "specific" binding defined as the binding displaceable by 30 μM 5-HT (A), 1.0 μM norzimeldine (*NZ*) (B), 30 μM 6-MeO-THβC (C), or 100 μM desipramine (*DMI*) (D). Corresponding estimations of B_{max} and K_d from individual experiments in A resulted in mean ±SE of 332 ± 16 fmol/mg of protein and 4.17 ± 1.07 nM, respectively (n = 19). B. For 1.0 μM norzimeldine, B_{max} was 349 ± 15 fmol/mg of protein and K_d was 4.47 ± 1.07 nM, not significantly different from 5-HT-displaceable binding (n = 22). C. For 30 μM 6-MeO-THβC, B_{max} was 439 ± 28 fmol/mg of protein and K_d was 5.49 ± 1.09 nM, not significantly different from 5-HT (n = 9). D. For 100 μM desipramine, B_{max} was 608 ± 42 fmol/mg of protein, $p < 0.01$ versus 5-HT, norzimeldine, and 6-MeO-THβC, and K_d was 6.68 ± 1.09 nM, $p < 0.01$ versus 5-HT and norzimeldine (n = 20). Depicted are means ± standard error for 9–22 experiments, the standard error values being <10%. [^{3}H]Imipramine concentration range was 1–30 nM. Not significant, $p > 0.05$; *, $p < 0.05$ **, $p < 0.01$ versus the values for specific binding with either 5-HT or norzimeldine as displacers (analysis of variance with à postiori testing of significance by Student-Newman-Keul's multiple range test).

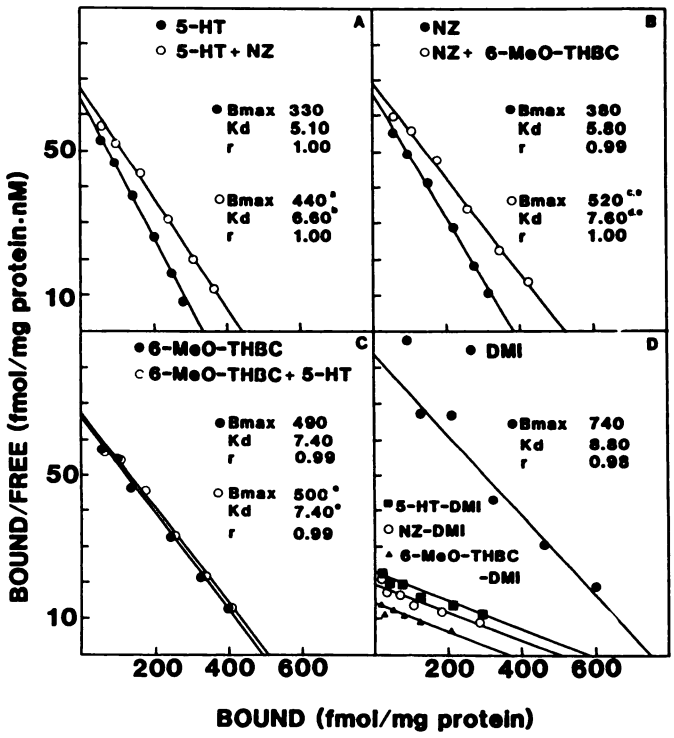

Fig. 4. Scatchard plots of [^{3}H]imipramine binding to rat cortical membranes. "Specific" binding is defined as the total binding in the absence of displacer minus the "nonspecific" binding in the presence of the drug(s) under test. The values are means of three experiments, and the standard error of each data point was <15%. The range of [^{3}H]imipramine concentrations used was 1–30 nM. "Nonspecific" binding was defined by the concentration of drug described for A–D. A. 5-HT (30 μM) (●), or 30 μM 5-HT + 1.0 μM norzimeldine (○). The Hill coefficient (n_H) for 5-HT-displaceable binding was 0.96 ± 0.02 (n = 3, mean ± SD). B. Norzimeldine (*NZ*) (1.0 μM) (●), n_H = 0.97 ± 0.01 (n = 3), or 1.0 μM norzimeldine + 30 μM 6-MeO-THβC (○). C. 6-MeO-THβC (30 μM) (●), n_H = 1.01 ± 0.00 (n = 3), or 30 μM 6-MeO-THβC + 30 μM 5-HT (○). D. Desipramine (*DMI*) (100 μM) (●), n_H = 1.02 ± 0.02 (n = 3). Also shown is nonspecific binding defined by 100 μM desipramine, but where the high affinity component was blocked by the presence of: 30 μM 5-HT (■), B_{max} 570 fmol/mg of protein, K_d 26 nM, r = 0.96; 1.0 μM norzimeldine (○), B_{max} 510 fmol/mg of protein, K_d 27 nM, r = 0.93; or (▲) 30 μM 6-MeO-THβC, B_{max} 370 fmol/ng of protein, K_d 27 nM, r = 0.93. Indicated are B_{max} (fmol/mg of protein), K_d (nM), and the correlation coefficient (r) for the linear regression. $^a p < 0.01$ vs. 5-HT; $^b p < 0.05$ vs. 5-HT; $^c p < 0.01$ vs. norzimeldine; $^d p > 0.05$ vs. norzimeldine; $^e p > 0.05$ vs. 6-MeO-THβC.

verified with significant differences in "specific" binding in five of six concentrations compared with 5-HT- and norzimeldine-displaceable binding (Fig. 3D).

When the combination of 5-HT (30 μM) and norzimeldine (1.0 μM) was used to define nonspecific binding, only a slight increase in K_d and B_{max} was noted (Fig. 4A). This indicates that most of the [^{3}H]imipramine-binding sites displaced by 30 μM 5-HT are identical to the sites with high affinity (<1.0 μM) for norzimeldine. The combination of 30 μM 6-MeO-THβC with either norzimeldine (1.0 μM) or 5-HT (30 μM) did not produce B_{max} or K_d changes compared to 6-MeO-THβC-displaceable binding alone, indicating that all of the binding displaced by norzimeldine or 5-HT is subsumed within the 6-MeO-THβC-displaceable binding (Fig. 4, B and C). Finally, the [^{3}H]imipramine binding displaced by 100 μM desipramine, but where the high affinity component was blocked by either 5-HT (30 μM), norzimeldine (1.0 μM), or 6-MeO-THβC (30 μM), respectively, is shown in Fig. 4D. Apparently, desipramine (100 μM) displaces additional binding with lower affinity for [^{3}H]imipramine than do 5-HT-displaceable sites. However, it should be noted that the B_{max} and K_d values indicated for those low affinity sites are but rough estimations, since the [^{3}H]imipramine concentrations are not saturating.

Na$^+$ dependency study. The ion dependency of the [^{3}H]imipramine binding was investigated by increasing the concentration of either NaCl or LiCl from 0 mM to 500 mM. In the absence of NaCl, the K_d of the binding displaced by 30 μM 5-HT was 51 nM, which subsequently decreased to 5.4 nM at 50 mM NaCl and 3.5 nM at 120 mM (Fig. 5A). There was a tendency for even lower K_d values at 250 mM and 500 mM NaCl, but statistically significant differences were not obtained. The enhanced binding is primarily due to an increase in affinity since no change in B_{max} was noted. A corresponding increase of LiCl (0–500 mM) did not produce any increase in 5-HT-displaceable binding. The binding at 120 mM LiCl is shown in Fig. 5A. This indicates that [^{3}H]imipramine binding displaced by 5-HT primarily is dependent on the presence of Na^+ ions but not Li^+ ions. Similar findings were noted for [^{3}H]imipramine binding displaced by 1.0 μM norzimeldine (Fig. 5B). In contrast, 100 μM desipramine displaced the [^{3}H]imipramine binding in the absence of ions, with K_d of 13 ± 1 nM and B_{max} of 320 ± 120 fmol/mg of protein (n = 3, mean ± SD). This binding fraction remained with increasing concentrations of LiCl; at 120 mM, K_d was 11 ± 2 nM and B_{max} was 280 ± 190 fmol/mg of protein (n = 4); at 500 mM, K_d was 19 ± 2 nM and B_{max} was 310 ± 80 fmol/mg of protein (n = 2).

Lesion studies. The location of [^{3}H]imipramine-binding

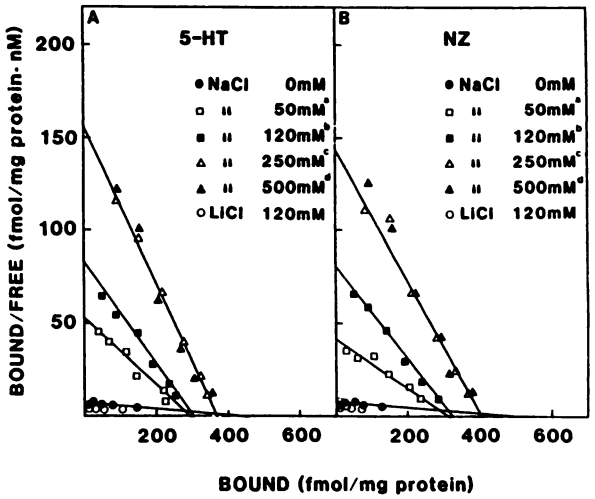

Fig. 5. Na^+ dependency of [^{3}H]imipramine binding. In saturation experiments with [^{3}H]imipramine (1–30 nM), the concentration of NaCl and LiCl, respectively, was increased from 0 mM to 500 mM (see Experimental Procedures). Depicted are Scatchard plots on the binding with increasing NaCl concentrations. Also shown is the binding at 120 mM LiCl (○), which was not different from the binding at 50, 250, or 500 mM LiCl (not shown in the figure). Binding was displaced by 30 μM 5-HT (A) or 1.0 μM norzimeldine (B). A. 5-HT (30 μM) at: NaCl, ○ mM (●), B_{max} 400 fmol/mg of protein, K_d 51 nM, r = 0.89, n = 4; 50 mM (□), B_{max} 290 fmol/mg of protein, K_d 5.4 nM, r = 0.95, n = 4; 120 mM (■), B_{max}, 290 fmol/mg of protein, K_d 3.5 nM, r = 0.99, n = 8; 250 nM (△, regression line indicated), B_{max} 370 fmol/mg of protein, K_d 2.4 nM, r = 1.00, n = 4; or 500 mM (▲, regression line not indicated), B_{max} 380 fmol/mg of protein, K_d 2.4 nM, r = 0.98, n = 4; or LiCl, 120 mM (○), B_{max} and K_d not determined. B. Norzimeldine (1.0 μM) at: NaCl, 0 mM (●), B_{max} 470 fmol/mg of protein, K_d 70 nM, r = 0.70, n = 4; 50 mM (□), B_{max} 320 fmol/mg of protein, K_d 7.7 nM, r = 0.97, n = 4; 120 mM (■), B_{max} 320 fmol/mg of protein, K_d 4.0 nM, r = 1.00, n = 8; 250 mM (△, regression line indicated), B_{max} 400 fmol/mg of protein, K_d 2.8 nM, r = 1.00, n = 4; or 500 mM (▲, regression line not indicated), B_{max} 390 fmol/mg of protein, K_d 2.4 nM, r = 0.98, n = 4; or LiCl, 120 mM (○), B_{max} and K_d not determined. Each data point represents the mean of the number of assays indicated above, the standard error being <15%. No significant differences in B_{max} were noted; the following was obtained with respect to K_d: $^a p < 0.01$ vs. 0 mM NaCl; $^b p < 0.05$ vs. 50 mM NaCl; $^c p > 0.05$ vs. 120 mM NaCl; $^d p > 0.05$ vs. 250 mM NaCl.

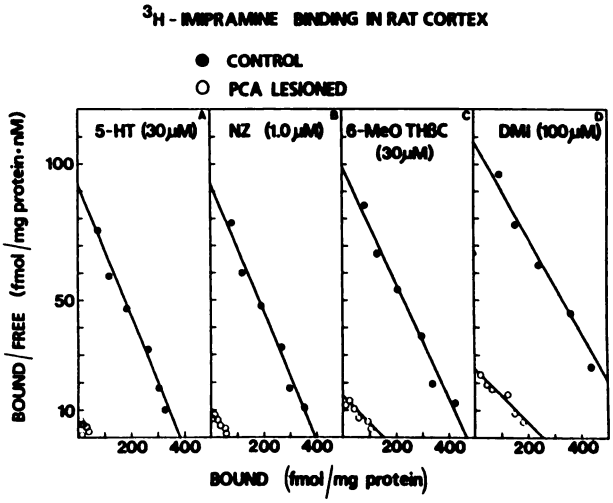

Fig. 6. [^{3}H]Imipramine binding in PCA-lesioned and control rat cortex. Rats were injected intraperitoneally twice (24 hr interval) with PCA (10 mg/kg). Control animals were treated similarly but with physiological saline instead of PCA. Lesioned and control animals were decapitated 7 days after final injection, whereafter the brain tissue was either assayed for [^{3}H]5-HT and [^{3}H]NA uptake or stored at −70° until binding experiments were performed (see Experimental Procedures). [^{3}H]Imipramine binding was performed using concentrations between 1 and 30 nM and "specific" binding was defined as the binding displaceable by: A, 5-HT (30 μM), B, norzimeldine (*NZ*) (1.0 μM); C, 6-MeO-THβC (30 μM); and D, desipramine (*DMI*) (100 μM). [^{3}H]Imipramine binding data presented in the figure represent the mean of three separate experiments, the standard error for each data point being <10%. The following B_{max} (fmol/mg of protein) and K_d (nM) values were obtained (values for controls within parentheses). A. Binding too low to estimate in lesioned animals (B_{max} 380, K_d 4.4 for control). B. B_{max} 75 (395), K_d 7.9 (4.2). C. B_{max} 150 (470), K_d 10.8 (4.7). D, B_{max} 250 (620), K_d 10.1 (5.7). The effects of PCA lesions were checked by uptake experiments as described in Experimental Procedures. [^{3}H]5-HT uptake was reduced to 17 ± 1% of control (n = 3), whereas [^{3}H]NA uptake was unaffected, 99 ± 9% of control (n = 3) (means ± SE).

sites was studied in lesion experiments (Fig. 6). Rats were injected intraperitoneally with PCA, which selectively abolishes 5-HT nerve terminals. This was indicated by an 83% reduction in 5-HT uptake without significant change in NA uptake (see legend to Fig. 6). In lesioned animals, the [^{3}H]imipramine binding displaced by 5-HT (30 μM) and norzimeldine (1.0 μM), respectively, was reduced to 20% of controls (Fig. 6, A and B). The binding competed by 6-MeO-THβC (30 μM) was 30% of control in lesioned animals, whereas desipramine (100 μM)-displaceable [^{3}H]imipramine binding was reduced to 40% of controls (Fig. 6, C and D). The absolute reduction in binding was about the same in all four cases, 310–360 fmol/mg of protein.

Regional distribution. The regional distribution of [^{3}H]imipramine-binding sites in the rat brain revealed the highest binding capacity in the hypothalamus and the striatum (Table 1). When 5-HT (30 μM) and norzimeldine (1.0 μM) were used to define specific binding, similar B_{max} and K_d values were obtained. However, 6-MeO-THβC (30 μM) gave higher B_{max} and K_d values and, with desipramine (100 μM), this was even more pronounced. The V_{max} and K_m values for the regional distribution of [^{3}H]5-HT and [^{3}H]NA uptake are also presented in Table 1. The correlation between 5-HT uptake and [^{3}H]imipramine binding was significant when 5-HT, norzimeldine, and 6-MeO-THβC, respectively, were used as displacers (Fig. 7, A–C), but nonsignificant for desipramine (Fig. 7D). Signifi-

TABLE 1

Regional distribution of [^{3}H]imipramine binding and of the uptake of [^{3}H]5-HT and [^{3}H]NA in rat brain

"Specific" [^{3}H]imipramine binding (1–30 nM) was defined by either 30 μM 5-HT, 1.0 μM norzimeldine (NZ), 30 μM 6-MeO-THβC, or 100 μM desipramine (DMI), corresponding to B_{max} (fmol/mg of protein) and K_d (nM) values in the columns below. Binding data represent the mean of three separate experiments, the standard error for each data point being <10%. Binding and uptake experiments were performed as described in Experimental Procedures. The synaptosomal uptake of [^{3}H]5-HT and [^{3}H]NA is presented as V_{max} (pmol/g of wet weight tissue/min) and K_m (nM) values. The same synaptosomal preparations from five rats were used for the [^{3}H]5-HT and [^{3}H]NA uptake. Each value was obtained from one experiment. Correlations between binding and uptake are presented in Fig. 7.

	[^{3}H]Imipramine binding								Uptake			
	5-HT		NZ		6-MeO-THBC		DMI		5-HT		NA	
	B_{max}	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}	K_d	V_{max}	K_m	V_{max}	K_m
FC[a]	291	4.38	304	4.48	353	4.79	681	6.49	190	53	68	87
PC	240	3.55	265	4.10	344	5.11	804	8.24	151	65	52	57
HY	432	4.15	480	4.61	523	4.90	974	3.82	356	75	157	156
S	413	4.46	429	4.53	494	4.96	847	8.60	288	66	ND[b]	
HC	366	4.73	404	5.59	424	5.09	814	8.90	221	68	139	173

[a] FC, frontal cortex; PC, parietal cortex; HY, hypothalamus; S, striatum; HC, hippocampus.
[b] ND, not determined.

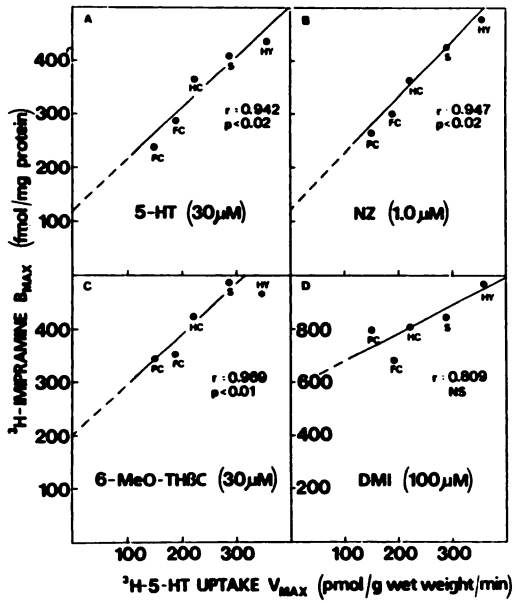

Fig. 7. Correlations between the regional distribution of [^{3}H]imipramine binding and [^{3}H]5-HT uptake. "Specific" [^{3}H]imipramine binding (1–30 nM) was defined by: A, 5-HT (30 μM); B, norzimeldine (*NZ*) (1.0 μM); C, 6-MeO-THβC (30 μM); or D, desipramine (*DMI*) (100 μM). Binding data represent the mean of three separate experiments, the standard error being <10%. Binding and uptake experiments were performed as described in Experimental Procedures. Indicated are B_{max} (fmol/mg of protein) and V_{max} values (fmol/g of wet weight/min), the correlation coefficients (r), and corresponding level of significance (p) obtained by Pearson's product moment correlation test. *PC*, parietal cortex, *FC*, frontal cortex; *HC*, hippocampus; *S*, striatum, *HY*, hypothalamus. The [^{3}H]imipramine binding was also tested against [^{3}H]NA uptake, $n = 4$. A, $r = 0.975$, $p < 0.05$; *B*, $r = 0.983$, $p < 0.02$; *C*, $r = 0.931$, not significant. D, $r = 0.739$, not significant (*NS*). The correlation between [^{3}H]5-HT and [^{3}H]NA uptake was $r = 0.86$, not significant ($n = 4$). *, B_{max} = 523 fmol/mg of protein.

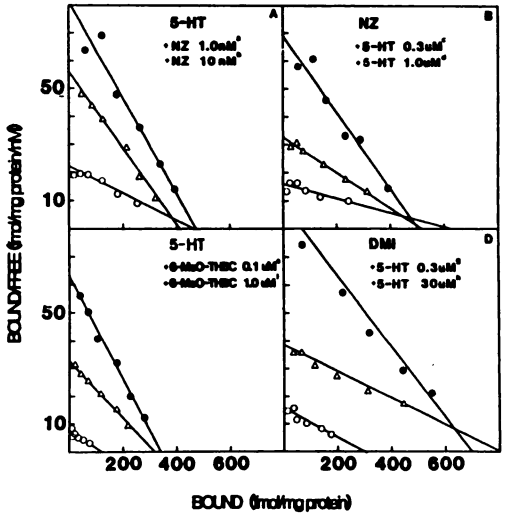

Fig. 8. Drug inhibition of [^{3}H]imipramine binding. The [^{3}H]imipramine binding (1–30 nM) was performed in the presence of two concentrations of inhibiting agents. The concentration of the additional inhibiting agent was chosen from displacement curves in Fig. 1. The data represent the mean values from four to nine separate experiments, the standard error for each data point being <10%. A. Specific binding was defined by 30 μM 5-HT (●) B_{max} 480 fmol/mg of protein, K_d 5.9 nM, $r = 0.97$, with the addition of 1.0 nM norzimeldine (△), B_{max} 420 fmol/mg of protein, K_d 7.3 nM, $r = 0.99$, K_i 4.2 nM, or 10 nM norzimeldine (○), B_{max} 460 fmol/mg of protein, K_d 21 nM, $r = 0.96$, K_i 3.9 nM. B. Specific binding was defined by 1.0 μM norzimeldine (*NZ*) (●), B_{max} 500 fmol/mg of protein, K_d 7.2 nM, $r = 0.97$, plus 0.3 μM 5-HT (△), B_{max} 520 fmol/mg of protein, K_d 16 nM, $r = 0.99$, K_i 250 nM, or 1.0 μM 5-HT (○), B_{max} 550 fmol/mg of protein, K_d 35 nM, $r = 0.95$, K_i 260 nM. C. Specific binding was defined by 30 μM 5-HT (●), B_{max} 340 fmol/mg of protein, K_d 5.5 nM, $r = 1.00$, plus 0.1 μM 6-MeO-THβC (△), B_{max} 310 fmol/mg of protein, K_d 9.7 nM, $r = 1.00$, or 1.0 μM 6-MeO-THβC (○), B_{max} 130 fmol/mg of protein, K_d 17 nM, $r = 1.00$. D. Specific binding was defined by 100 μM desipramine (*DMI*). (●), B_{max} 700 fmol/mg of protein, K_d 7.9 nM, $r = 0.96$, plus 0.3 μM 5-HT (△), B_{max} 790 fmol/mg of protein, K_d 21 nM, $r = 0.98$, or 30 μM 5-HT (○), B_{max} 300 fmol/mg of protein, K_d 20 nM, $r = 0.96$. Significance of K_d/B_{max} values, respectively, is indicated as follows: $^a p > 0.05/> 0.05$ vs. 5-HT only; $^b p < 0.01/> 0.05$ vs. +1.0 nM NZ; $^c p < 0.05/> 0.05$ vs. NZ only; $^d p < 0.05/ > 0.05$ vs. +0.3 μM 5-HT; $^e p > 0.05/> 0.05$ vs. 5-HT only; $^f p > 0.05/< 0.01$ vs. +0.1 μM 6-MeO-THβC; $^g p < 0.05/> 0.05$ vs. DMI only; $^h p < 0.05/< 0.05$ vs. DMI only.

cant correlations between [^{3}H]imipramine binding and noradrenaline uptake was found, but this can be explained by the notable positive correlation between 5-HT and NA uptake (see legend to Fig. 7).

Drug inhibition studies. The nature of pharmacological interactions by 5-HT, norzimeldine, 6-MeO-THβC, and desipramine with [^{3}H]imipramine-binding sites was also studied. When [^{3}H]imipramine binding defined by 30 μM 5-HT was performed in the presence of low concentrations of norzimeldine (1.0 nM and 10 nM), increases in K_d without changes in B_{max} were obtained, which would suggest that norzimeldine inhibits the binding in a competitive manner (Fig. 8A). In analogy, [^{3}H]imipramine binding displaced by norzimeldine (1.0 μM), and in the presence of low concentrations of 5-HT (0.3 μM and 1.0 μM), displayed competitive inhibition (Fig. 8B). When 0.1 μM 6-MeO-THβC was added to 5-HT-displaceable [^{3}H]imipramine binding, no change in either K_d or B_{max} was noted. At 1.0 μM 6-Meo-THβC, a pronounced decrease in B_{max} was obtained, suggesting a more complex interaction (Fig. 8C). This was also the case when the [^{3}H]imipramine binding displaced by 100 μM desipramine was inhibited by low concentrations of 5-HT (Fig. 8D).

Inhibition of [^{3}H]5-HT uptake by imipramine. The inhibition of the uptake of [^{3}H]5-HT by imipramine was competitive (Table 2). The K_i value for imipramine was calculated from nine experiments to be 24 ± 15 nM (mean ± SD).

TABLE 2

Inhibition of synaptosomal [^{3}H]5-HT uptake by imipramine

Synaptosomes were prepared from rat cerebral cortices and uptake experiments were performed as described in Experimental Procedures. V_{max} and K_m values were determined from Eadie-Hofstee plots by linear regression analysis. The following V_{max} (pmol/g/min) and K_m (nM) values were obtained (mean ± standard deviation; number of experiments in parentheses).

		V_{max}	K_m	
Control		100 ± 16	34 ± 7	(5)
Imipramine	25 nM	94 ± 3	47 ± 2	(2)
	50 nM	104 ± 3	105 ± 23	(4)
	100 nM	104 ± 19	168 ± 84	(3)

Discussion

Numerous studies of the binding of [^{3}H]imipramine to membranal fragments of rodent brain have demonstrated a close relationship between the imipramine-binding site and the 5-HT uptake system. Thus, there was an excellent correlation between the potencies of a variety of compounds to inhibit the uptake of 5-HT and to displace the specific binding of imipramine (4). The regional distribution of endogenous 5-HT parallels that of the imipramine-binding site (3). Lesions of the

serotonergic neurons decrease the binding of [^{3}H]imipramine and the 5-HT levels in several brain regions (15–17). However, in spite of the close association noted between the imipramine binding and the 5-HT uptake, some other observations have led Sette *et al.* (6) to suggest that "[^{3}H]imipramine binds to a site associated with the 5-HT uptake system but different from either the substrate recognition site for 5-HT or the site of action of the non-tricyclic inhibitors of neuronal uptake of 5-HT." This observation was based on the findings that 5-HT and non-tricyclic 5-HT uptake inhibitors inhibited [^{3}H]imipramine in a complex manner with Hill slopes far below unity (6). As a consequence of this model of the relationship between the imipramine-binding site and the 5-HT uptake site, the existence of an endogenous ligand (endocoid) for the imipramine-binding site was proposed (17–20). This hypothetical endocoid is supposed to have a regulating function of the 5-HT uptake mechanism, and speculations of a chemical structure closely related to 6-MeO-THβC have been made (11).

The proposed model of the association between the [^{3}H]imipramine-binding site and the 5-HT uptake mechanism is obviously valid only if it is assumed that one class of binding sites for imipramine exists. Several studies have revealed a second type of binding site with low affinity for imipramine (K_d 200–700 nM) (7–9). Marcusson *et al.* (10) found that only part (50% in rat frontal cortex) of the high affinity [^{3}H]imipramine binding was sensitive to protease treatment. This part of the binding could also be discriminated by the 5-HT uptake inhibitor, norzimeldine. In contrast, when desipramine was used as displacer, this site could *not* be discriminated from another binding fraction, resistant to protease treatment and not related to 5-HT uptake (10). Obviously, when 100 μM desipramine is used to define specific binding, the [^{3}H]imipramine binding represents a non-homogeneous class of binding sites. These findings prompted the reevaluation of the relationship between the 5-HT uptake and the high affinity binding site for [^{3}H]imipramine discriminated by norzimeldine.

The results obtained in the present study demonstrate that the [^{3}H]imipramine-binding site, as defined with norzimeldine (1 μM) or 5-HT (30 μM), is of protein nature and is located on serotonin terminals. Furthermore, the data suggest that this site is identical to the site binding 5-HT for transport. This is based on several observations considered together.

1. This class of [^{3}H]imipramine binding is localized on the serotonergic nerve terminals, since lesioning of these terminals with PCA, a selective serotonergic neurotoxin in the rat cerebral cortex (21, 22), reduced the [^{3}H]imipramine binding and the 5-HT uptake to the same extent (80%), whereas only a 60% reduction of the [^{3}H]imipramine binding was obtained when defined with 100 μM desipramine as displacer. Sette *et al.* (17), who defined the specific binding with desipramine, observed a smaller effect on the [^{3}H]imipramine binding than on the 5-HT uptake or the endogenous 5-HT level after chemical denervation of 5-HT neurons with 5,7-dihydroxytryptamine. Thus, the [^{3}H]imipramine-binding site not displaced by norzimeldine (1 μM) or 5-HT (30 μM) does not seem to be localized on serotonergic nerve terminals.

2. The good correlation found between the number of [^{3}H]imipramine-binding sites as defined with norzimeldine or 5-HT and the V_{max} values for 5-HT uptake in the brain regions tested supports a relationship between these two parameters. When the regression lines in Fig. 7 were extrapolated toward zero level of 5-HT uptake, the [^{3}H]imipramine binding defined by either 5-HT or norzimeldine was 6-fold lower compared with desipramine as displacer, with the intercepts with the ordinate approaching the origin. Thus, 5-HT- and norzimeldine-displaceable [^{3}H]imipramine binding appears to be more directly associated to the 5-HT uptake mechanism.

3. The [^{3}H]imipramine-binding site as defined with 5-HT or norzimeldine was shown to be proteinaceous, which is in accordance with the protein nature of the 5-HT uptake carrier (23).

4. The transport of 5-HT by the neuronal uptake mechanism is sodium dependent. The carrier, Na^+, and 5-HT appear to form a ternary complex on the outside of the neuron membrane, and this complex is translocated through the membrane (24). The sodium ions cannot be substituted by lithium ions in this process. Like the 5-HT uptake, the [^{3}H]imipramine-binding site defined with norzimeldine or 5-HT has an absolute requirement for sodium ions. These ions increase the affinity (lowered K_d) without effect on the B_{max}. Similarly, Na^+ increases the affinity of the 5-HT binding to the uptake carrier (lowered K_m) without changing V_{max} (24, 25). In contrast, the [^{3}H]imipramine binding component, displaceable by 100 μM desipramine in the absence of NaCl or in the presence of LiCl, shows the same characteristics as the desipramine-displaceable binding where the high affinity sites had been blocked by either 5-HT or norzimeldine. This low affinity site has previously been shown to be protease resistant and not related to the 5-HT uptake mechanism (10).

5. 5-HT inhibited this class of high affinity [^{3}H]imipramine-binding sites competitively with a K_i value of 0.3 μM. Since the rate-limiting step in the transport of 5-HT through the membrane most likely is the diffusion of the carrier-5-HT complex and the return of the carrier and not of the formation of the 5-HT-carrier complex, a lower K_m value (approximately 0.05 μM) for 5-HT uptake is to be expected. This "discrepancy" has been one of the major arguments for a two-site model of the 5-HT uptake and imipramine-binding complex. However, the binding of 5-HT to the surface of the 5-HT uptake complex, i.e., the substrate recognition site, indicated by 5-HT-displaceable [^{3}H]imipramine binding, is only the first step of the uptake process and therefore cannot be identical to the physiological process involved in the uptake of 5-HT. Furthermore, besides having some attachment to the carrier in common with 5-HT, the noncomplementary part of the imipramine molecule may have additional bonds to the 5-HT carrier, which would contribute to a higher K_i value for 5-HT on [^{3}H]imipramine displacement compared to the K_m for 5-HT uptake.

6. Imipramine inhibited competitively the uptake of 5-HT in a cortical synaptosomal preparation with a K_i value of 24 nM. The competitive interaction suggests that imipramine binds to the substrate recognition site for 5-HT uptake. However, the K_i value is about 5 times higher than the K_d value for the [^{3}H]imipramine binding displaceable by 5-HT. This discrepancy between the K_d and K_i values is most likely explained by the decreased affinity of [^{3}H]imipramine binding at higher incubation temperatures. Indeed, Dumbrille-Ross *et al.* (26) found a K_d value of 24 nM for [^{3}H]imipramine binding in rat cerebral cortex at 37° (but 8 nM at +4°), in concordance with the K_i value of imipramine on the inhibition of 5-HT uptake reported in the present study.

7. Norzimeldine inhibited competitively the [^{3}H]imipramine binding displaceable by 5-HT (K_i 4 nM). Norzimeldine is also a competitive inhibitor of the neuronal 5-HT uptake with a K_i value of about 40 nM in synaptosomes prepared from rat hypothalamus (27). The competitive interactions with both

[^{3}H]imipramine binding and 5-HT uptake suggest a common site of action, namely, the substrate recognition site for 5-HT uptake. The discrepancy between K_i and K_m values for binding and uptake may have the same explanations as described above for imipramine.

The simplest and most likely explanation of these findings is that imipramine binds with high affinity to the same site in the 5-HT transporter that binds 5-HT for transport and also other tricyclic and non-tricyclic 5-HT uptake inhibitors. Analysis of the structural requirements for inhibition of the neuronal 5-HT uptake by various compounds has shown the possibility to superimpose models of 5-HT and most of the 5-HT uptake inhibitors (28). Thus, the imipramine binding producing inhibition of 5-HT uptake would be to the substrate recognition site for 5-HT uptake.

When regarding the whole 5-HT uptake mechanism, it is likely that the 5-HT carrier may exist in different conformations when binding 5-HT at the outside and when ejecting 5-HT at the inside of the membrane. However, the latter conformation does not appear to bind the inhibitors since it is induced by high concentrations of potassium (24). Finally, even though it appears less likely from the present data, it cannot be ruled out that the uptake inhibitors including imipramine bind to a site which allosterically interacts in a competitive manner with the 5-HT-binding site in the carrier protein.

6-MeO-THβC was included in the study since it has been proposed to be structurally related to a potential endogenous ligand for the imipramine-binding site (12). This compound displaced [^{3}H]imipramine binding from the same site as did norzimeldine and 5-HT, but also from an additional site in common with desipramine but not with norzimeldine or 5-HT. It cannot, from these data, be determined whether 6-MeO-THβC has a regulatory function on the site binding 5-HT for uptake. However, most of the binding of 6-MeO-THβC is to the substrate recognition site for neuronal 5-HT uptake, which is in line with the reported competitive inhibition by 6-MeO-THβC of 5-HT uptake (29).

In conclusion, the data now presented evidently raise the possibility that the binding site for 5-HT uptake inhibitors is identical to the substrate recognition site for neuronal 5-HT uptake. This single-site model of the 5-HT uptake complex can be described as a receptor protein that binds 5-HT as well as tricyclic and non-tricyclic 5-HT uptake inhibitors and the putative endogenous ligand 6-MeO-THβC to a common site. However, depending on the concentrations used, both the antidepressants and, to a lesser extent, 6-MeO-THβC bind to non-proteinaceous recognition sites not related to the 5-HT uptake complex. When 100 μM desipramine is used to define "specific" binding, 50% of the binding (in cortex) is to these recognition sites (10), the function of which, at present, is unknown, but which may represent lipophilic membrane components (30).

Acknowledgments

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