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Comparison of [^{3}H]Paroxetine and [^{3}H]Cyanoimipramine for Quantitative Measurement of Serotonin Transporter Sites in Human Brain

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Previous studies have demonstrated that [^{3}H]paroxetine and [^{3}H]cyanoimipramine ([^{3}H]CN-IMI) are highly selective ligands for the serotonin (5-HT) transporter. Using membrane preparation from the putamen, we confirmed that in human brain [^{3}H]paroxetine labeled with high affinity one class of site associated with the 5-HT transporter. [^{3}H]CN-IMI labeled two classes of sites in human brain. The one displaceable by 5-HT and with high affinity to 5-HT uptake inhibitors accounts for about 60% of the [^{3}H]CN-IMI binding and, presumably, is associated with the 5-HT transporter. From the competition experiments, citalopram was selected to define [^{3}H]CN-IMI binding to the 5-HT transporter in tissue sections because of its high selectivity to 5-HT transporter sites. A good correlation of the regional distribution patterns for [^{3}H]CN-IMI and [^{3}H]paroxetine was found using quantitative autoradiography. However, [^{3}H]paroxetine underestimated high concentrations of the 5-HT transporter comparing to [^{3}H]CN-IMI. This is likely to be due to the higher specific activity of [^{3}H]CN-IMI. There is a good correlation between the regional distribution of 5-HT transporter sites labeled with either [^{3}H]paroxetine or [^{3}H]CN-IMI and the density of serotonergic innervation. This suggests that the brain areas that receive numerous serotonergic afferents, such as the hypothalamus and basal forebrain, might be common targets of these antidepressant drugs. Pharmacologic similarity of the sites labeled by both ligands as well as their similar distribution in the brain suggests that both antidepressant drugs interact with the same protein, thereby eliciting a similar neurochemical response.
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Serotonin transporter sites have been attracting considerable attention from researchers for many years, because they are thought to be involved in pathological processes associated with major depression. Early reports utilizing [^{3}H]imipramine as a radioligand for the 5-HT transporter showed decreased density of [^{3}H]imipramine sites in the frontal cortex and hypothalamus of depressed suicide victims (Stanley et al. 1982) and in the occipital cortex and hippocampus of depressed subjects (Perry et al. 1983). This was confirmed by another group using [^{3}H]citalopram as a radioligand (Leake et al. 1991). Two reports have also observed reduced concentrations of 5-HT transporter sites in the frontal cortex of patients with a primary diagnosis of schizophrenia (Laruelle et al. 1993; Joyce et al. 1993). As discussed elsewhere, this reduction may also be related to the high probability of suicide in schizophrenic patients (Laruelle et al. 1993; Joyce 1994). Drugs used to treat de-

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pression, antidepressants, are believed to modify monoamine transmission. The mechanism of this effect is unknown. Classic tricyclic antidepressants bind to noradrenaline as well as to 5-HT transporter sites with varying selectivity, making the identification of the site of therapeutic action unclear. Recently, several highly efficacious antidepressants have been introduced, such as paroxetine, fluoxetine, sertraline, which bind with high potency and selectivity to 5-HT transporter sites. Their effectiveness has provided firm support for a hypothesis that the blockade of 5-HT uptake sites by antidepressant drugs is predominantly responsible for their therapeutic activity (see Owen and Nemeroff 1994).

The ability to quantify 5-HT transporter sites in different regions of the brains derived from normal cases and from patients with neuropsychiatric illnesses is important for the further verification of this hypothesis (Crow et al. 1984; Gross-Isseroff et al. 1989; Joyce et al. 1993; Laruelle et al. 1993; Stanley et al. 1982). [^{3}H]imipramine was the first ligand to be extensively used to characterize the distribution of 5-HT transporter sites in the brain and their alterations in neuropsychiatric illnesses. However, binding studies with [^{3}H]imipramine yielded contradictory results, because the ligand labels two sites, only one of which seems associated with the 5-HT transporter (D'Amato et al. 1987). The introduction of more selective and potent 5-HT transporter inhibitors allowed for the development of radioligands that selectively labeled 5-HT transporter sites. For example, [^{3}H]paroxetine has been shown to label selectively and with high affinity a single type of site associated with the 5-HT transporter in the rat brain (DeSouza and Kuyatt 1987; Hrdina et al. 1990; Cheetham et al. 1993). This was determined by examining the displacement of this compound from tissue sections and membrane preparations by drugs with differing affinity and selectivity for the 5-HT transporter. In the rat brain the [^{3}H]paroxetine binding to membranes prepared from discrete brain regions correlated well with regional 5-HT content, thereby serving as a good marker for 5-HT innervation density (Dewar et al. 1992; Dewar et al. 1990). [^{3}H]Cyanoimipramine ([^{3}H]CN-IMI) has also been shown to label selectively and with high affinity a single class of sites associated with the 5-HT transporter in the rat brain sections (Kovachich et al. 1988; Soucy et al. 1994). The regional distribution of the [^{3}H]CN-IMI binding sites in the rat brain is in close agreement with the distribution of the binding sites labeled with [^{3}H]indalpine, [^{3}H]imipramine, and [^{3}H]paroxetine (Kovachich et al. 1988; Hrdina et al. 1990; Gobbi et al. 1990; DeSouza and Kuyatt 1987; Kovachich et al. 1992). However, several inconsistencies in the binding of different radioligands have been noted, for example, much lower density for [^{3}H]paroxetine than for [^{3}H]CN-IMI in the hypothalamus and amygdala. In addition, it was reported that in the human brain [^{3}H]paroxetine bound to multiple sites (Backstrom et al. 1989). This finding was not supported in other studies (Plenge et al. 1990; Laruelle et al. 1988). The [^{3}H]paroxetine binding sites are heterogeneously distributed in the human brain, with the nucleus raphe, hypothalamus, and substantia nigra displaying the highest density (Backstrom et al. 1989; Laruelle et al. 1988; Plenge et al. 1990). However, the density varies considerably among individuals (Plenge et al. 1990). The autoradiographic comparison of the distribution of the [^{3}H]paroxetine and [^{3}H]imipramine binding sites in the human brain showed that the ligands exhibited similar distribution patterns, with the highest densities found in the nucleus raphe (Cortés et al. 1988). The autoradiographic distribution of the [^{3}H]CN-IMI binding to 5-HT transporter sites in several regions of human brain has been reported to be different from that identified with [^{3}H]paroxetine or [^{3}H]imipramine (Joyce et al. 1983). These inconsistencies may reflect differences in the pharmacology of sites labeled by [^{3}H]paroxetine and [^{3}H]CN-IMI. In the present study we have characterized the pharmacology of the binding of [^{3}H]paroxetine and [^{3}H]CN-IMI to membranes derived from the human putamen of normal individuals. We then directly compared their regional binding patterns using quantitative autoradiography.

METHODS

Preparation of Tissue Sections

Brain tissue was obtained from the left or right hemisphere of each of 15 (nine men, six women) control cases from the Hospital of the University of Pennsylvania (HUP). The control cases had no known history of neurologic, neurovascular, or psychiatric diseases and died of natural causes. Routine gross and microscopic neuropathologic examination (John Q. Trojanowski, M.D., Ph.D., Director of Medical Pathology, HUP) confirmed the lack of neuropathologic abnormalities in the control tissue. The mean age (±SD) at death for controls was 67.6 ± 4.3 years. The mean post-mortem interval (PMI) between death and procurement of the brain tissue was 11.9 ± 1.9 hours. Causes of death included myocardial ischemia, candida bronchopneumonia, respiratory failure, pulmonary hemorrhage, and acute myocardial infarction. No tissue was included from cases where suicide or probable suicide was considered the cause of death. Hemisphere sections were dissected into 2- to 3-cm thick blocks, rapidly frozen at −40°C, and kept frozen at −70°C. At the time of the experiment the tissue was brought to −20°C, and 20-μm thick sections were cut from the blocks of tissue with a Frigocut cryostat. The sections were dried under reduced pressure and stored at −70°C until needed.

Membrane Binding Studies

Total membrane fraction of the putamen was used in the binding experiments. Tissue was homogenized in

10 v (w/v) of ice-cold 50 mmol/L Tris HCI buffer, pH 7.5, and centrifuged for 20 minutes at 40,000 *g*. The resulting pellet was washed with the same buffer and then resuspended in 0.5 mol/L sucrose buffered with 50 mmol/L Tris HCI, pH 7.5. The suspension was centrifuged for 1 hour at 40,000 *g*, and the pellet resuspended in the 50 mmol/L Tris HCI buffer, pH 7.5, containing 120 mmol/L NaCl and 5 mmol/L KCl at a final concentration of 100 mg tissue (wet weight) /ml.

For saturation analysis, each test tube contained 200 µl of membrane suspension, 100 µl of [^{3}H]paroxetine or [^{3}H]cyanoimipramine, and 100 µl of buffer or displacing drug. For competition experiments, each test tube contained 300 µl of membrane suspension, 150 µl of a radioligand, and 150 µl of displacing drug. There were three total and two nonspecific tubes for each concentration point in the saturation experiments, and all competition studies were performed in triplicate. Saturation analysis was performed with 0.02-4 nmol/L of [^{3}H]paroxetine or 0.015 to 3 nmol/L of [^{3}H]CN-IMI. Nonspecific binding was defined as that in the presence of 100 µmol/L desipramine or 150 µmol/L 5-HT. Incubation was initiated by the addition of tissue, carried out for 90 minutes at room temperature with [^{3}H]paroxetine or for 4 hours at 4°C for [^{3}H]CN-IMI. The reaction was terminated by addition of 5 ml of ice-cold 50 mmol/L Tris HCl buffer (pH 7.5). The membrane suspension was rapidly filtered through GB/F filters. The filters were washed three times more with 5 ml of buffer and then put into scintillation vials containing 4 ml of scintillation fluid. Vials were left overnight and then counted in Rackbeta counter (LKB, Sweden).

Quantitative Autoradiographic Studies

The slide-mounted tissue sections were preincubated in 50 mmol/L Tris HCl buffer, pH 7.5, containing 120 mmol/L NaCl and 5 mmol/L KCl. After preincubation, the sections were dried by vacuum aspiration and then incubated with 0.25 nmol/L of [^{3}H]paroxetine in the same buffer for 2 hours at room temperature. Upon completion of the incubation the sections were washed in the same Tris HCl buffer three times for 1 hour at 4°C. The sections were then briefly dipped in ddH_2O and dried. The procedure for incubation with [^{3}H]CN-IMI was performed as described by Joyce et al. (1993) using a 0.5 nmol/L concentration of [^{3}H]CN-IMI. Briefly, the sections were preincubated in the 50 mmol/L Tris HCl buffer, pH 7.5, containing 150 mmol/L NaCl for 10 minutes at 4°C. Incubation with [^{3}H]CN-IMI was carried out at 4°C for 24 hours. Upon completion of the incubation the sections were washed in the same buffer two times for 1 hour, then washed in ddH_2O for 10 seconds, and dried. For both radioligands 1 µmol/L citalopram was used to define nonspecific binding. To generate autoradiograms, the sections along with ^{3}H plastic standards (American Radiolabeled Chemicals, St. Louis, MO) were opposed to ^{3}H-Hyperfilm (Amersham) for 45 days at room temperature.

Data Analysis

The saturation and competition data were analyzed using FITSAT and FITCOMP programs from PROPHET software package. K_i values were calculated from IC_{50}s according to the equation of Chang and Prusoff (Munson and Rodbard 1988). Autoradiograms were analyzed using a Macintosh image analysis system and Brain for Macintosh software (Drexel University, Philadelphia) that permitted the conversion of optical density of the autoradiographic images to biological units (fmol/mg protein). The adjacent brain sections were stained for Nissl and acetylcholinesterase (AChE) as described in detail elsewhere (Joyce et al. 1986) to facilitate the identification of the brain structures. The binding data were then analyzed by one-way ANOVA with brain area as a main factor, and the binding densities in the different regions were compared using post hoc Fisher's protected LSD test.

Chemicals

[^{3}H]paroxetine was obtained from Du Pont (New England Nuclear) with a specific activity of 18.75 Ci/mmol. [^{3}H]cyanoimipramine was obtained from American Radiolabeled Chemicals (St. Louis, MO) with a specific activity of 85 Ci/mmol. Nonradioactive paroxetine was a gift of Beecham Pharmaceuticals Research Division, Philadelphia, PA. Citalopram, fluoxetine, desipramine were a generous gift of Dr. Alan Frazer (University of Texas, San Antonio). All other chemicals were purchased from Sigma (St. Louis, MO).

RESULTS

Saturation and Competition Experiments

The saturation analysis of [^{3}H]paroxetine binding to membranes of the human putamen, with desipramine to define nonspecific binding, showed that the ligand bound to a single class of sites with high affinity (Figure 1*a*). The K_d value, as determined by FITSAT, was 0.316 ± 0.05 nmol/L, and the B_{max} was 4.54 ± 0.25 pmol/g wet tissue. Saturation analysis for [^{3}H]paroxetine, with 150 mmol/L of 5-HT to define nonspecific binding, yielded a K_d value 0.34 ± 0.07 nmol/L and a B_{max} of 4.57 ± 0.3 pmol/g tissue. The data obtained with either method to define nonspecific binding are in good agreement. The results of the saturation experiments suggest that [^{3}H]paroxetine labels a single class of sites associated with 5-HT transporter. In parallel experiments with another 5-HT transporter site ligand, [^{3}H]CN-IMI, it was determined

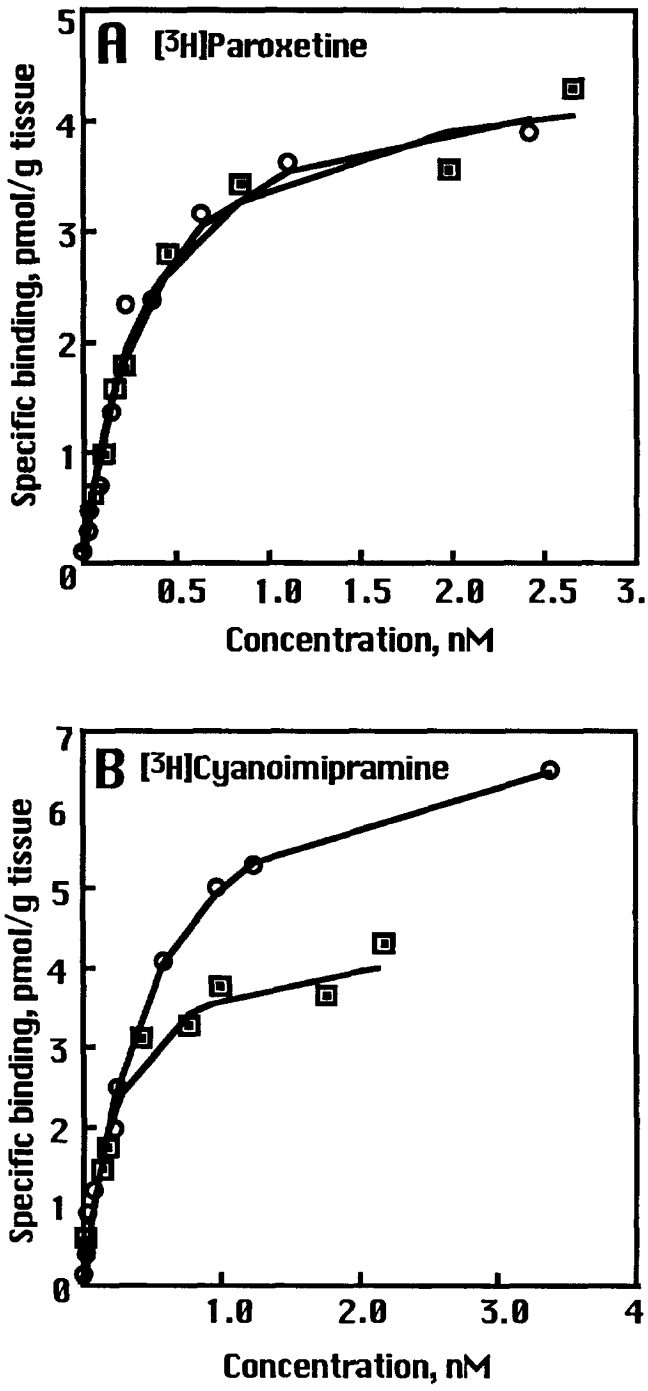


Figure 1. Saturation curves for [^{3}H]paroxetine **(A)** and [^{3}H]CN-IMI **(B)** binding to membrane preparations of the human putamen. Nonspecific binding was defined using 100 μmol/L desipramine or 150 μmol/L 5-HT. The binding conditions were as described in the Methods section. Non-specific binding with: ○ 100 μM desipramine; ▣ 150 μM 5-HT.

that the B_{max} for [^{3}H]CN-IMI was more than 50% higher than that for [^{3}H]paroxetine (Figure 1*b*). The saturation analysis of [^{3}H]CN-IMI binding to membranes of the human putamen, with nonspecific binding defined with 100 μmol/L of desipramine, yielded a K_d value of 0.49 ± 0.06 nmol/L and a B_{max} of 7.4 ± 0.34 pmol/g tissue. The saturation analysis of [^{3}H]CN-IMI binding with the nonspecific binding defined by 150 μmol/L of 5-HT yielded K_d value 0.23 ± 0.08 nmol/L and a B_{max} of 4.47 ± 0.45 pmol/g tissue. The B_{max} obtained with 5-HT as the displacer is almost identical to that of [^{3}H]paroxetine and more than 50% lower than the B_{max} obtained with desipramine as a displacing agent. The results suggest that [^{3}H]CN-IMI, in contrast to [^{3}H]paroxetine, binds with similar affinity to more than one class of binding sites in membranes of the human putamen.

The results of the competition analysis for [^{3}H]paroxetine are shown in Figure 2*a*. The potent inhibitors of the 5-HT transporter citalopram and fluoxetine were also potent displacers of [^{3}H]paroxetine binding in membranes from the human putamen. In all cases, the displacement curves fit to a single-site model with Hill coefficient close to 1. The order of potency was citalopram > fluoxetine > desipramine. The results showed that paroxetine was a selective ligand that labeled a single class of sites pharmacologically indistinguishable from 5-HT transporter sites. The competition studies with [^{3}H]CN-IMI supported the conclusion that the ligand labeled at least two types of binding sites. All competition curves (Figure 2*b*) fitted significantly better to a two-site than to a one-site model. The K_i values of the competing compounds for the high affinity component were in a good agreement with their K_i values for the competition with [^{3}H]paroxetine. The proportions of the sites displaced with high affinity by citalopram, fluoxetine, desipramine, and paroxetine (70.3%, 77.5%, 68.0%, and 65.4%, respectively) were similar to each other and to the proportion of sites displaced by 5-HT (60.4%). Because of the high selectivity and potency for displacement of [^{3}H]paroxetine and [^{3}H]CN-IMI from 5-HT transporter sites, citalopram was chosen as a displacer for the definition of specific binding in the autoradiographic experiments.

Quantitative Autoradiographic Studies

The percentage of specific binding for [^{3}H]CN-IMI in tissue sections varied from more than 95% in the areas of high concentration of sites, like the hypothalamus and basal forebrain, to 50% to 40% in regions with a low concentration of sites such as the cortex. Most of the values fell in the range of 80% to 85% of specific binding. [^{3}H]Paraoxetine displayed higher nonspecific binding than [^{3}H]CN-IMI. The range of specific binding was from 80% in the hypothalamus to 20% in the hippocampal formation. The average percentage of specific binding was about 50% to 55%.

[^{3}H]Paroxetine and [^{3}H]CN-IMI binding sites were heterogeneously distributed throughout the brain. Tables 1–6 present the average values determined from single concentration autoradiographic experiments with [^{3}H]par-

oxetine and [^{3}H]CN-IMI. Figure 3 shows pseudocolor photographs of individual sections representing various brain regions. As illustrated in Figure 3 a–l, the distributions of [^{3}H]paroxetine and [^{3}H]CN-IMI binding sites in the various brain structures were very similar. In the basal ganglia, moderate concentrations of both [^{3}H]paroxetine and [^{3}H]CN-IMI binding sites were found in the rostral and caudal caudate nucleus, putamen, claustrum, and globus pallidus (Table 1). The ventral putamen and nucleus accumbens exhibited higher concentrations of sites than more dorsal regions (Figure 3 a–e). The region of the nucleus basalis of Meynert, identified on Nissl-stained sections and sections processed for AChE histochemistry, displayed high density of binding sites for each radioligand (Figure 3 b, e). The bed nucleus of stria terminalis and the vertical division of the nucleus of diagonal band of Broca also showed intense labeling (Figure 3 a–e). Within regions of the rostral midbrain the highest concentrations existed in the central gray (Table 2). The different regions of the substantia nigra, paranigral and parabrachial pigmented nuclei exhibited moderate concentrations of sites (Figure 3 i, l). Most nuclei of the thalamus (Figure 3 c, f) had low to intermediate concentrations of the binding sites labeled with both ligands, except for the nuclei along the midline (Table 3). The area with relatively high labeling includes the nucleus parafascicularis, nucleus medialis dorsalis, and nucleus anteroventralis. Generally, the concentration of binding sites was higher in the anteromedial than in the posterolateral part of the thalamus.

The nuclei of the hypothalamus (Figure 3 b, e) showed consistently high concentrations of binding sites (Table 4). However, some differences in patterning of [^{3}H]paroxetine and [^{3}]CN-IMI binding were noticeable. The highest level of [^{3}H]CN-IMI binding was observed in the paraventricular nucleus and dorsal hypothalamic area, and somewhat lower levels in the tuberomamillar nucleus, the dorsomedial nucleus, and lateral hypothalamic area. For [^{3}H]paroxetine, the binding was the highest in the lateral tuberal nucleus and paraventricular nucleus, and slightly lower concentrations in the dorsomedial nucleus and dorsal hypothalamic area. Similar patterns of binding for [^{3}H]paroxetine and [^{3}H]CN-IMI existed in the amygdala and hippocampus (Tables 5 and 6). The concentration of sites in most nuclei of the amygdala (Figure 3 g, i) was at an intermediate level, with higher densities found in the anterior amygdaloid area, ventral cortical amygdaloid nucleus, and amygdalopiriform transitional area. A low concentration of binding sites was observed in the hippocampal formation (Figure 3 h, k). The pyramidal cell layer exhibited low concentrations of sites in all hippocampal fields. The concentration of sites was the lowest in the CA1 hippocampal field and presubiculum, and the binding was slightly higher in the perirhinal and entorhinal cortex. The concentration of the [^{3}H]CN-IMI binding

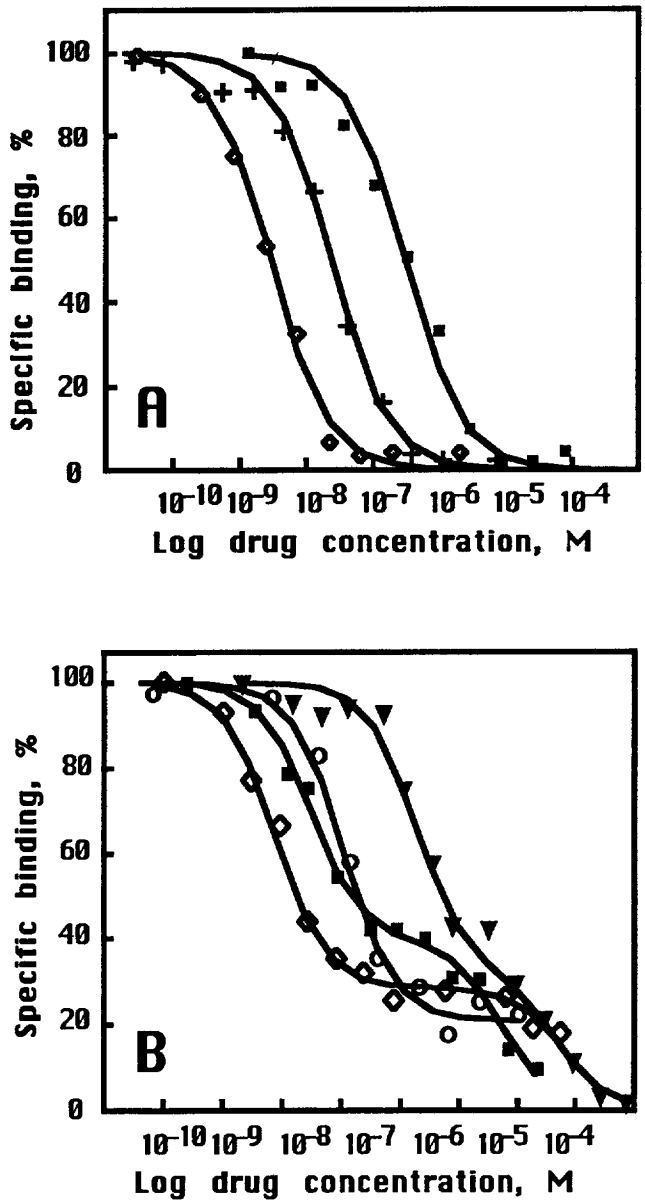


Figure 2. Inhibition of [^{3}H]paroxetine **(A)** (◇ citalopram; + fluoxetine; ■ desipramine) and [^{3}H]CN-IMI **(B)** (◇ citalopram; ■ paroxetine; ○ fluoxetine; ▼ desipramine) binding by 5-HT uptake inhibitors. The binding conditions were as described in the Methods section. All competition curves were best fit by assuming a one-site model for [^{3}H]paroxetine and a two-site model with at least $p < .005$ for [^{3}H]CN-IMI. Ki values for the competition with [^{3}H]paroxetine were 1.1. nmol/L for citalopram, 9 nmol/L for fluoxetine, and 118 nmol/L for desipramine. For the competition with [^{3}H]CN-IMI, Ki values for the high affinity site were 0.48, 2.26, 7.95, and 154 nmol/L for citalopram, paroxetine, fluoxetine, and desipramine, respectively. Ki values for the low affinity site were 1.6 μmol/L for paroxetine, 30 μmol/L for desipramine, 37 μmol/L for citalopram, and more than 100 μmol/L for fluoxetine.

Table 1. Concentrations of [^{3}H]Cyanoimipramine and [^{3}H]Paroxetine Binding Sites (fmols/mg protein) in the Subdivisions of the Basal Ganglia and Basal Forebrain

	[^{3}H]CN-IMI Binding	*n*	[^{3}H]Paroxetine Binding	*n*	Ratio[a]
Rostral striatum					
Dorsal caudate nucleus	200.5 ± 62.0	7	180.2 ± 55.9	6	1.6 ± 0.6
Ventral caudate nucleus	323.6 ± 65.9	7	151.8 ± 103.8	6	2.1 ± 0.6
Dorsal putamen	217.0 ± 47.9	7	276.0 ± 68.8	7	0.8 ± 0.2
Ventral putamen	304.8 ± 60.5	7	330.0 ± 90.2	7	1.4 ± 0.8
Nucleus accumbens	465.0 ± 102.5	5	296.0 ± 27.5	5	2.0 ± 0.9
Caudal striatum					
Caudate nucleus	305.7 ± 36.9	6	240.6 ± 64.8	5	2.0 ± 0.7
Putamen	277.6 ± 54.3	6	226.7 ± 48.7	5	1.6 ± 0.8
Globus pallidus, external	205.3 ± 52.7	6	203.6 ± 59.5	4	1.0 ± 0.3
Globus pallidus, internal	389.9 ± 52.7	4	196.0 ± 43.0	4	2.2 ± 0.4
Claustrum	187.8 ± 49.2	7	265.6 ± 71.1	5	1.3 ± 0.4
Nucleus basalis of Meynert	1264.0 ± 91.5	4	424.4 ± 140.1	3	2.6 ± 1.1
Bed nucleus of stria terminalis	682.1 ± 140.0	5	556.9 ± 67.5	5	1.2 ± 0.3
N. of diagonal band Broca	815.6 ± 30.3	3	598.5 ± 92.3	3	1.4 ± 0.2

[a] The ratio is concentration of [3H]CN-IMI/[3H]paroxetine sites.

sites was the highest in the stratum lacunosum-moleculare of all fields. The binding of [^{3}H]paroxetine in the hippocampal formation followed essentially the same pattern. Low specific activity of [^{3}H]paroxetine combined with its high nonspecific binding in the human brain precluded reliable determination of the concentration of sites in the cortex with this radioligand. The data obtained with [^{3}H]CN-IMI showed low and relatively uniform concentration of sites in the various areas of the cortex (Table 7), with slightly higher concentration in the precentral and postcentral gyri than in the prefrontal cortex. The cingulate cortex exhibited the highest binding and did not differ between the anterior and posterior divisions.

The correlation between the mean binding values for [^{3}H]paroxetine and [^{3}H]CN-IMI in various brain regions is depicted in Figure 4. Overall lower binding of [^{3}H]paroxetine as compared to [^{3}H]CN-IMI is due to the difference in the concentrations used. However, the relationship between the binding values is best described by a second order polynomial model, rather than linear model, which may be due mostly to the tendency of [^{3}H]paroxetine to underestimate high values comparing to [^{3}H]CN-IMI. Figure 5 presents the binding profiles for the ligands in the different brain subdivisions examined. The graphs representing the relative profiles of each radioligand again indicate their high correlation. The tendency for [^{3}H]paroxetine to underestimate the concentrations of sites in the regions with high densities of the [^{3}H]CN-IMI binding is evident. [^{3}H]Paroxetine binding is also less sensitive to regional variations in concentrations of sites, as revealed by the smoother binding profiles ob-

Table 2. Concentrations of [^{3}H]Cyanoimipramine and [^{3}H]Paroxetime Binding Sites (fmols/mg protein) in the Subdivisions of the Midbrain

	[^{3}H]CN-IMI Binding	*n*	[^{3}H]Paroxetine Binding	*n*	Ratio[a]
Red nucleus	320.4 ± 60.7	6	207.2 ± 63.2	5	1.9 ± 0.7
Substantia nigra					
Pars compacta	509.7 ± 176.9	5	324.1 ± 32.3	5	1.9 ± 0.9
Pars reticulata	194.6 ± 43.6	5	219.4 ± 47.0	5	0.9 ± 0.1
Pars lateralis	379.8 ± 68.1	6	271.3 ± 85.8	6	2.0 ± 1.0
Paranigral nucleus	472.5 ± 160.9	5	397.5 ± 57.0	5	1.2 ± 0.4
Parabrachial pigmented nucleus	390.8 ± 158.2	5	295.4 ± 85.7	5	1.5 ± 0.4
Nucleus Raphe					
Caudal linear nucleus	413.1 ± 68.9	4	362.3 ± 100.2	3	1.4 ± 0.7
Rostral linear nucleus	169.4 ± 40.6	3	233.5 ± 57.6	2	0.3 ± 0.2
Central grey	744.2 ± 279.2	3	584.7 ± 167.4	3	1.7 ± 1.1

[a] The ratio is concentration of [^{3}H]CN-IMI/[^{3}H]paroxetine sites.

Table 3. Concentration of [^{3}H]Cyanoimipramine and [^{3}H]Paroxetine Binding Sites (fmols/mg protein) in the Subdivisions of Thalamus

	[^{3}H]CN-IMI Binding	*n*	[^{3}H]Paroxetine Binding	*n*	Ratio[a]
Anterior:					
N. anteroventralis	235.3 ± 21.5	4	264.4 ± 39.0	3	0.9 ± 0.1
Medial:					
N. medialis dorsalis	299.3 ± 12.0	5	241.7 ± 46.5	3	1.3 ± 0.2
Central:					
N. centrum medianum	252.4 ± 46.1	3	186.7 ± 23.0	4	1.3 ± 0.2
N. parafascicularis	398.1 ± 86.9	5	309.6 ± 71.3	4	1.2 ± 0.3
Dorsal:					
N. lateralis posterior	170.0 ± 18.9	4	177.8 ± 36.9	3	0.9 ± 0.3
Ventral:					
N. ventralis posterolateralis	114.8 ± 20.7	5	149.2 ± 46.9	3	0.4 ± 0.1
Zona inserta	701.4 ± 190.1	3	362.4 ± 47.6	3	1.9 ± 0.3

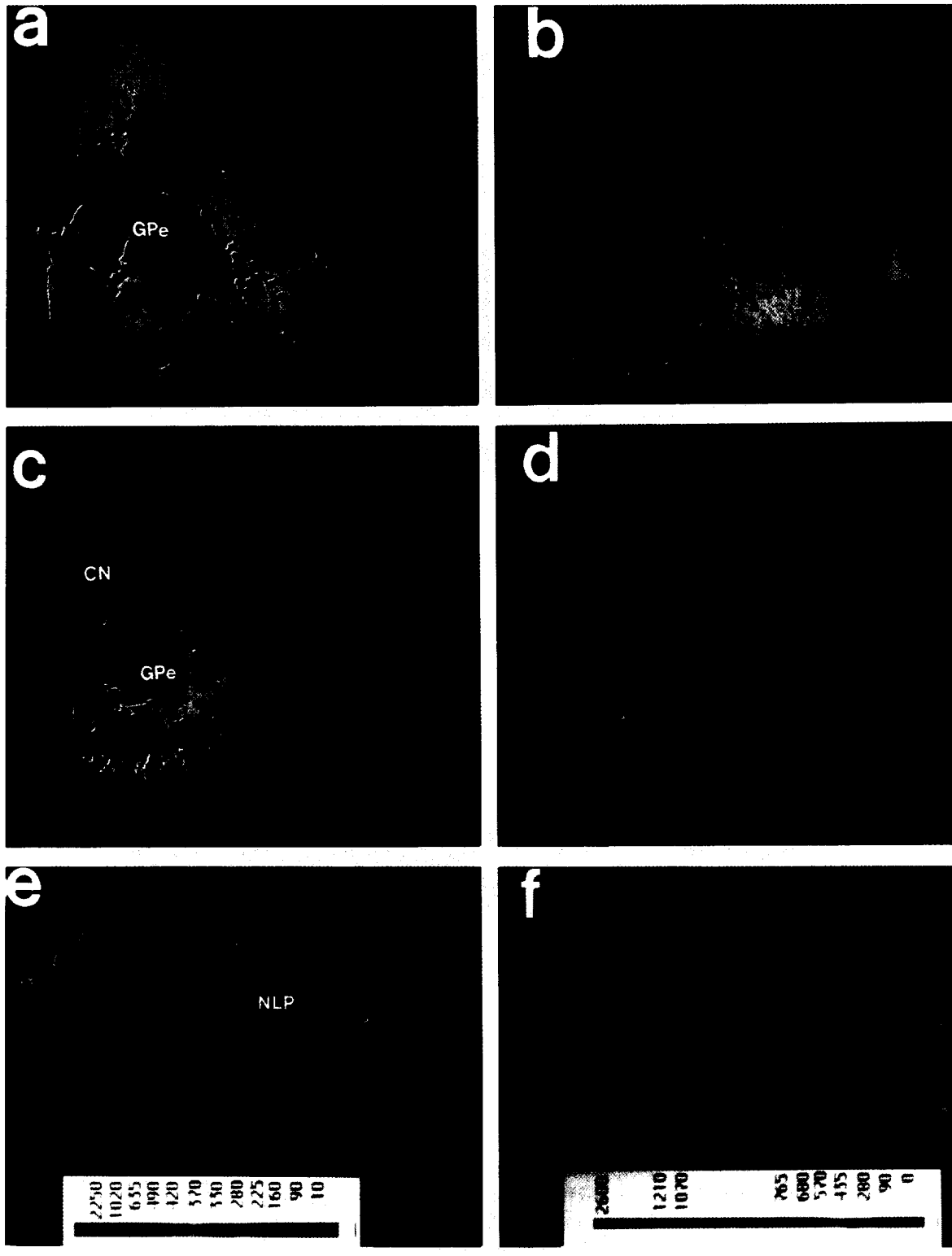
[a] The ratio is concentration of [^{3}H]CN-IMI/[^{3}H]paroxetine sites.

tained with [^{3}H]paroxetine as compared with [^{3}H]CN-IMI. This was particularly noticeable in the regions with a high degree of heterogeneity in the concentration of the [^{3}H]CN-IMI binding sites such as the basal ganglia and midbrain. In the amygdala, where all binding densities were at intermediate level, the binding profiles for both ligands are virtually identical. In the hypothalamus, where [^{3}H]CN-IMI binding values were similarly high, the concentrations of sites appeared to have been underestimated by [^{3}H]paroxetine to a similar degree.

DISCUSSION

Previous studies have demonstrated that [^{3}H]paroxetine and [^{3}H]CN-IMI are potent and highly selective ligands for the 5-HT transporter in rat brain (Kovachich et al. 1988; Hrdina et al. 1990; Dewar et al. 1992). In our experiments with human brain, [^{3}H]paroxetine bound to one class of sites with high affinity. Serotonin transporter inhibitors were potent displacers of [^{3}H]paroxetine binding, and all competition curves were best fit by assuming a single site. Our results showed that, in contrast to the rat brain, [^{3}H]CN-IMI labeled two types of sites in the human putamen when desipramine was used as a displacer to define binding to the 5-HT transporter. Only one site was identifiable when binding to the 5-HT transporter was defined as that displaced by a saturating concentration of 5-HT. There was approximately a 50% reduction in the B_{max} value when 5-HT was used to determine binding to the 5-HT transporter instead of desipramine. This can be explained by assuming that [^{3}H]CN-IMI labeled at least two sites with similar affinity, and only one of the sites is identical to the 5-HT transporter. The competition experiments supported that conclusion. All competition curves for displacement of [^{3}H]CN-IMI by 5-HT transporter inhibitors exhibited a best fit by a two-site model. The Ki values for the high affinity component were in good agreement with the respective Ki values for their competition with [^{3}H]paroxetine. The class of sites with high affinity to 5-HT and 5-HT transporter inhibitors accounted for more than 60% of the [^{3}H]CN-IMI binding. Because of the very low affinity of citalopram and fluoxetine for the sites not associated with the 5-HT transporters, these compounds can be used to determine specific binding of [^{3}H]CN-IMI to the 5-HT transporter. Thus, 1 μmol/L citalopram displaced [^{3}H]CN-IMI selectively from the site related to the 5-HT transporter (more than 10,000-fold difference in K_is for the low and high affinity sites). Therefore, this concentration of citalopram was used to define 5-HT transporter-specific binding in the autoradiographic studies for both [^{3}H]paroxetine and [^{3}H]CN-IMI. Under these conditions the values obtained with each ligand were in good agreement and were consistent with that for the 5-HT transporter site.

Direct comparison of the binding values in the brain regions is not possible because of the different concentrations of the ligands used in the study and the difference in their K_ds. The degree of correspondence was examined by comparing their relative binding patterns in the brain subdivisions studied by autoradiography. The profiles of the relative binding for each radioligand were remarkably similar in all brain subdivisions examined (Figure 5). However, [^{3}H]CN-IMI showed more pronounced differences between brain regions than did [^{3}H]paroxetine (Figure 5). Regression analysis indicated that the relationship between the [^{3}H]CN-IMI and [^{3}H]paroxetine mean binding values obtained for different brain areas fitted best to a second-order polynomial model and not to a linear model. [^{3}H]paroxetine tended to underestimate the concentration of sites in



the densely labeled regions as compared to [^{3}H]CN-IMI (Figure 4). Lower specific activity of the commercially available [^{3}H]paroxetine (almost five times lower than that of [^{3}H]CN-IMI) results in an overall lower degree of sensitivity and resolution. Given the high specific activity and high ratio of total to nonspecific binding of [^{3}H]CN-IMI in human brain sections, it seems to be a ligand of choice to measure 5-HT transporter binding sites in the human brain. However, it is necessary to select carefully the displacer for determination of 5-HT

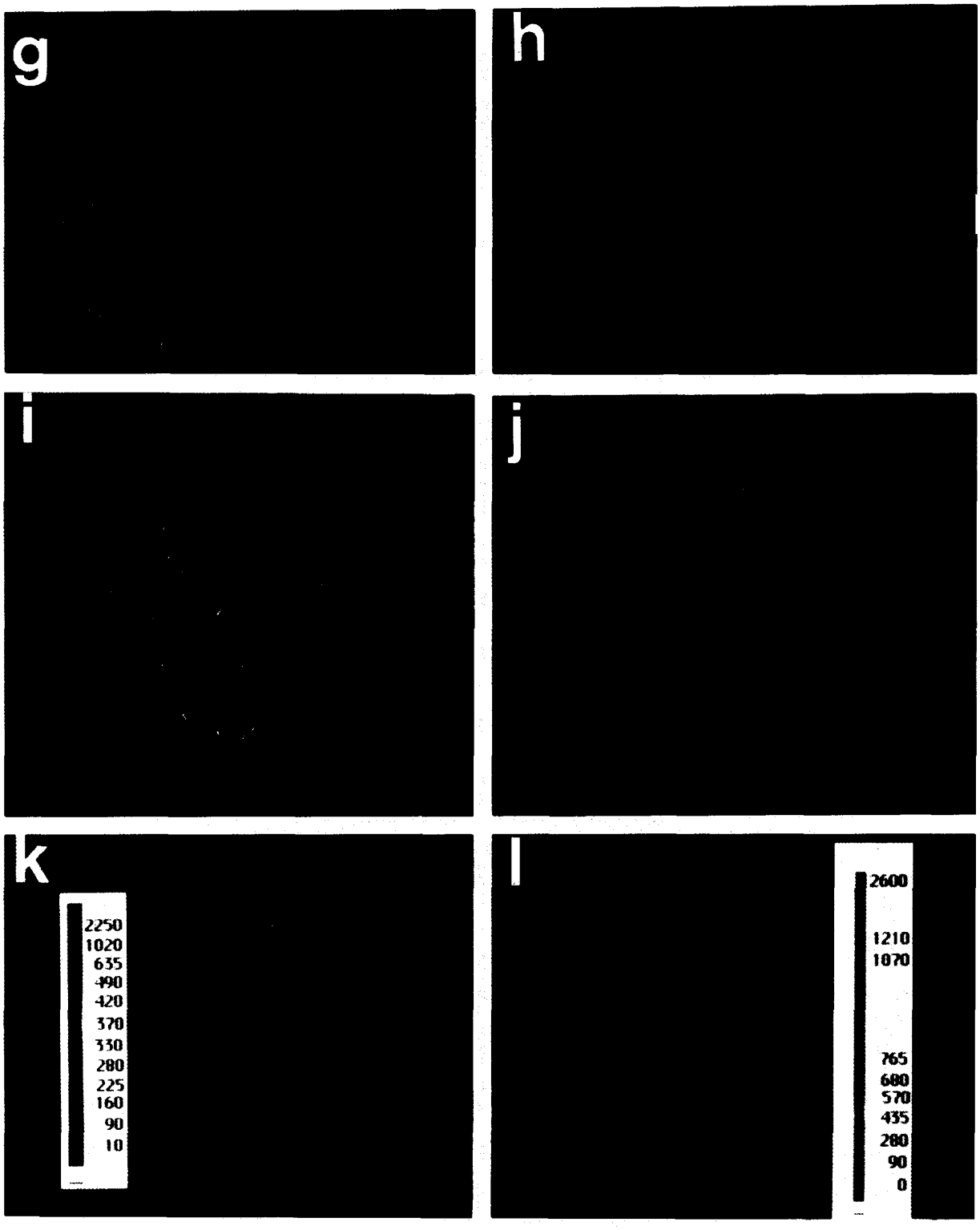


Figure 3. Pseudocolor pictures of the specific [^{3}H]CN-IMI (*left panels*: **a, c, e, g, i, k**) and [^{3}H]paroxetine (*right panels*: **b, d, f, h, j, l**) binding to individual brain sections representative for the subdivisions examined. Software Brain for Macintosh, version 4, was used to perform aligning and subtraction of the images of nonspecific from the images of total binding on pixel by pixel basis to obtain the images of specific binding. Gray values were assigned to a range of 32 pseudocolors based on a calibration curve. The range of values in fmols/mg protein and their corresponding pseudocolors are shown on the scale bars in panels **e, f, k, i**. The scale bars and pseudocolor images for [^{3}H]CN-IMI and [^{3}H]paroxetine were adjusted to ensure that similar colors represented similar values (in fmol/mg) for both ligands.

Table 4. Concentrations of [^{3}H]Cyanoimipramine and [^{3}H]Paroxetine Binding Sites (fmols/mg protein) in the Subdivisions of the Hypothalamus

	[^{3}H]CN-IMI Binding	*n*	[^{3}H]Paroxetine Binding	*n*	Ratio[a]
Supraoptic nucleus	903.3 ± 87.5	3	305.1 ± 54.5	3	2.9 ± 0.7
Dorsomedial nucleus	1177.7 ± 169.3	3	509.5 ± 97.2	3	3.6 ± 0.4
Paraventricular nucleus	1459.9 ± 180.5	3	517.4 ± 153.4	3	3.1 ± 0.4
Lateral hypothalamic area	1035.8 ± 60.3	3	373.6 ± 65.4	3	3.0 ± 0.4
Mamillary body	544.5	1	191.3	3	2.9
Tuberomammillary nucleus	1210.5	1	411.6	1	2.9
Lateral tuberal nucleus	1010.0	1	538.0	1	1.9
Dorsal hypothalamic area	1491.7	1	455.3	1	3.3
Periventricular nucleus	975.0	1	432.1	1	2.3

[a] The ratio is concentration of [^{3}H]CN-IMI/[^{3}H]paroxetine sites.

transporter-specific binding. Use of citalopram of fluoxetine to define specific binding assures the selectivity of [^{3}H]CN-IMI binding to the 5-HT transporter.

Our autoradiographic results also demonstrated a significant degree of heterogeneity in the concentrations of 5-HT transporter sites in different brain regions. Comparison of the regional differences in the concentrations of 5-HT transporter sites showed that the hypothalamus was enriched with 5-HT transporter sites, whereas the thalamus had low to moderate densities (except for the nuclei along the midline). The amygdala had moderate concentrations of the binding sites, except for the amygdalopiriform transitional area and the part of the extended amygdala, the bed nucleus of stria terminalis, which displayed high concentrations of 5-HT transporter sites. This region of high concentration of 5-HT transporter sites stretches rostrally into the basal forebrain, including the nucleus basalis of Meynert, ventral putamen, nucleus accumbens, and the nucleus of the diagonal band of Broca, without interruption. We have previously shown that the patches of high concentration of 5-HT transporter sites in the ventral putamen and nucleus accumbens are associated with the matrix compartment (Joyce et al. 1993). This is consistent with what has been observed for serotonergic fiber innervation of the basal ganglia and basal forebrain (Lavoie and Parent 1990; Tork and Hornung 1990). In the rostral midbrain the substantia nigra pars compacta and the central gray showed dense labeling, whereas the other structures had moderate binding densities. This is consistent with identification of 5-HT immunoreactive cell bodies in the central gray and substantia nigra of primates (Tork and Hornung 1990; Takahashi et al. 1986). The cortex and hippocampal formation had generally low concentrations of 5-HT transporter sites. The exceptions were the high binding observed in the stratum lacunosum-moleculare of the hippocampus, and moderate binding in the entorhinal and the perirhinal cortices. The 5-HT innervation to the hippocampus arises from both the dorsal and median raphe (Amaral and Campbell 1986; Kosofsky and Molliver 1987; Tork and Hornung 1990). Binding of [^{3}H]CN-IMI to 5-HT uptake sites is consistent with immunocytochemical studies of the patterning of 5-HT axons arising from the median raphe (Wilson and Molliver 1991b). In the primate cortex the density of 5-HT

Table 5. Concentrations of [^{3}H]Cyanoimipramine and [^{3}H]Paroxetine Binding Sites (fmols/mg protein) in the Subdivisions of Amygdala

	[^{3}H]CN-IMI Binding	*n*	[^{3}H]Paroxetine Binding	*n*	Ratio[a]
Nucleus lateralis	330.6 ± 45.8	3	177.0 ± 59.0	3	2.6 ± 1.1
Nucleus basolateralis	464.1 ± 89.1	3	170.7 ± 10.8	3	2.8 ± 0.7
Nucleus basomedialis	331.5 ± 10.4	3	144.9 ± 21.1	3	2.4 ± 0.5
Anterior amygdaloid area	503.2 ± 25.5	2	197.5 ± 60.8	2	2.9 ± 1.0
Nucleus centralis	412.6 ± 33.7	2	154.2 ± 38.7	2	2.8 ± 0.5
N. vertical cortical Amygdaloid	480.2 ± 160.3	3	250.2 ± 68.4	3	1.9 ± 0.2
Amygdalopiriform Transition area	850.8 ± 54.3	3	255.4 ± 20.6	3	3.4 ± 0.3

[a] The ratio is concentration of [^{3}H]CN-IMI/[^{3}H]paroxetine sites.

Table 6. Concentrations of [^{3}H]Cyanoimipramine and [^{3}H]Paroxetine Binding Sites (fmols/mg protein) in the Subdivisions of the Hippocampal Formation

	[^{3}H]CN-IMI Binding	*n*	[^{3}H]Paroxetine Binding	*n*	Ratio[a]
Hippocampus					
Dentate gyrus, granular layer	110.2 ± 16.3	9	105.2 ± 28.7	9	1.2 ± 0.3
Dentate gyrus, molecular layer	89.5 ± 11.4	9	129.8 ± 16.5	9	1.1 ± 0.1
CA3 field (all laminae except s. lacunosum-moleculare	94.8 ± 14.8	9	137.5 ± 21.5	9	1.4 ± 0.4
CA2 field	150.0 ± 26.8	10	145.9 ± 75.3	10	1.2 ± 0.6
CA1 field	60.6 ± 9.9	10	73.7 ± 20.8	10	0.9 ± 0.4
Stratum lacunosum-moleculare (all fields)	249.8 ± 52.2	10	257.2 ± 50.7	10	1.1 ± 0.1
Parahippocampus					
Subiculum	99.3 ± 18.2	10	59.1 ± 18.5	10	1.4 ± 0.3
Presubiculum	71.8 ± 13.0	10	64.5 ± 17.3	10	0.8 ± 0.1
Parasubiculum	88.2 ± 17.7	8	75.3 ± 24.0	8	1.2 ± 0.2
Entorhinal cortex	143.0 ± 21.7	7	85.3 ± 17.9	7	1.6 ± 0.3
Perirhinal cortex	162.3 ± 38.6	10	116.7 ± 19.4	10	1.6 ± 0.4

[a] The ratio is concentration of [^{3}H]CN-IMI/[^{3}H]paroxetine sites.

immunoreactive fibers is relatively similar across all laminae in the frontal cortex with only slightly higher densities in layer I. In the motor and parietal cortex the fibers are denser in the supragranular layers (Tork and Hornung 1990; Wilson and Molliver 1991a). These data are consistent with the distribution of 5-HT uptake sites in the human cortex labeled with [^{3}H]CN-IMI. The density of 5-HT uptake sites was relatively uniform across cortical laminae with slightly more dense binding in the superficial layer.

This work supports and extends our previous autoradiographic findings with [^{3}H]CN-IMI (Joyce et al. 1993). The values obtained for the basal ganglia, cortex, and hippocampus are in good agreement with the previous results. This work extends the analysis to other regions and provides a detailed comparison with the highly selective ligand [^{3}H]paroxetine. The only published work, to the best of our knowledge, that reports the results of autoradiographic studies with [^{3}H]paroxetine and another 5-HT transporter ligand ([3H]imi-

Table 7. Concentrations of the [^{3}H]Cyanoimipramine Binding Sites (fmols/mg protein) in Subdivisions of the Cortex

	[^{3}H]Cyanoimipramine Binding (fmol/mg protein)	*n*
Prefrontal cortex		
Cingulate gyrus	107.5 ± 63.7	4
Superior frontal gyrus	33.7 ± 10.8	4
Middle frontal gryus	21.8 ± 7.0	4
Inferior frontal gyrus	28.6 ± 11.8	3
Precentral gyrus		
Cingulate gyrus	126.1 ± 22.5	6
Superior frontal gyrus	50.9 ± 7.4	6
Middle frontal gyrus	62.1 ± 17.2	7
Inferior frontal gyrus	64.1 ± 22.6	7
Postcentral gyrus		
Cingulate gyrus	126.5 ± 23.7	4
Superior frontal gyrus	83.0 ± 28.6	4
Middle frontal gyrus	53.7 ± 12.1	4
Inferior frontal gyrus	73.1 ± 33.5	4
Temporal lobe		
Superior temporal gyrus	82.3 ± 23.4	3
Middle temporal gyrus	61.2 ± 15.6	3
Inferior temporal gyrus	96.8 ± 23.2	3
Occipital lobe	107.3 ± 12.5	2

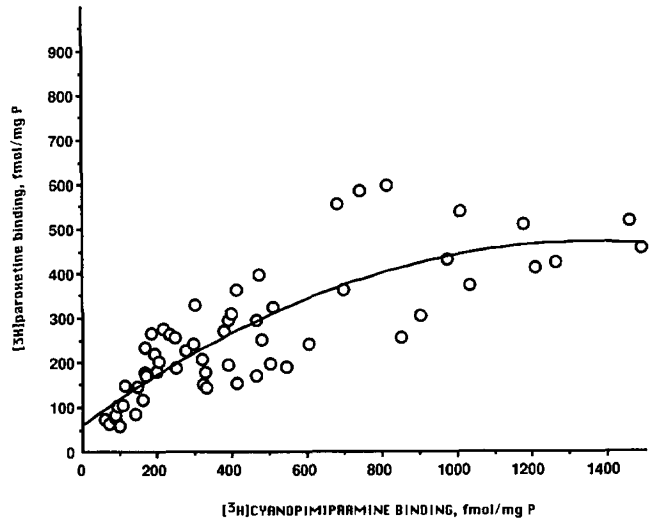


Figure 4. Correlation analysis of the mean binding values obtained with [^{3}H]paroxetine and [^{3}H]CN-IMI in different brain areas. The relationship is best described by second-order polynomial rather than linear function. The tendency of [^{3}H]paroxetine to underestimate high binding values as compared to [^{3}H]CN-IMI can be clearly seen. [$R = 0.679, p < .01$.]

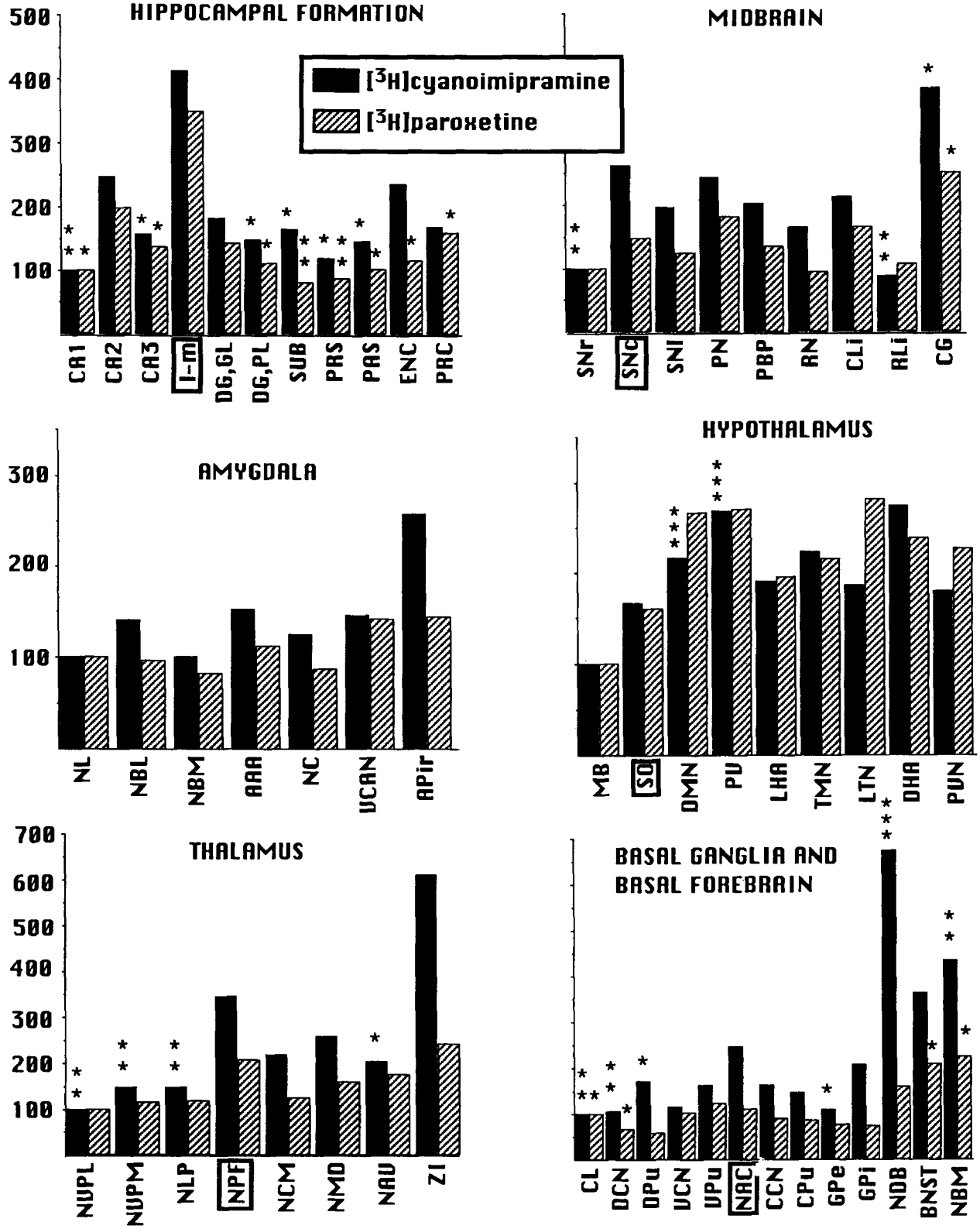


Figure 5. Relative binding profiles for [^{3}H]paroxetine and [^{3}H]CN-IMI in various brain regions. The values expressed as percentage of the binding in the regions with the lowest binding for [^{3}H]CN-IMI in each subdivision adjusted to 100% (*first bars on each graph*): CA1 field in the hippocampal formation, SNr in the midbrain, NL in the amygdala, MB in the hypothalamus, NVPL in the thalamus, and Cl in the basal ganglia. The data were analyzed by one-way ANOVA with brain region as main factor. The main effect was significant for [^{3}H]CN-IMI binding ($F = 61.9$ (5, 264), $p = .0001$) as well as for [^{3}H]paroxetine binding ($F = 18.3$ (5, 237), $p = .0001$). Asterisks denote statistically significant differences with $^*p < 0.05$, $^{**}p < .01$, and $^{***}p <$

pramine) in the human brain shows values reasonably similar to those obtained in the present study (Cortés et al. 1988). The direct comparison of the present results with that study is hampered by the different anatomic subdivisions examined in their work and the partial results with [^{3}H]paroxetine. However, the values reported here, and in the study of Cortés et al. (1988), are considerably higher (2 to 3 times or more) than previously found using membrane preparations from different areas of the human brain (Plenge et al. 1990; Backstrom et al. 1989; Laruelle et al. 1988). In our experiments, the binding values for the putamen membrane preparation were approximately 2 to 2.5 times lower than those obtained from the autoradiograms. The reason may be, as noted by Cortés et al. (1988), that there is a depletion of the sites with membrane preparations. It also may be that the autoradiograms provide higher degree of resolution, so that white matter, which inevitably contaminates membrane preparations, can be excluded. Cortés and associates (1988) also found a good agreement between the binding values obtained with [^{3}H]paroxetine and [^{3}H]imipramine (both ligands were used in saturating concentrations), as we did with [^{3}H]CN-IMI and [^{3}H]paroxetine. However, other investigators have found that the B_{max} values for [^{3}H]imipramine were almost twice as high as the values for [^{3}H]paroxetine in the human platelets (Nemeroff et al. 1994). Comparison of [^{3}H]imipramine binding with the binding of another ligand thought to be selective to 5-HT transporter sites, [^{3}H]citalopram, demonstrated that the binding patterns of the two ligands differed considerably in the human hypothalamus, hippocampus, and amygdala (Duncan et al. 1992). The distribution of binding sites for [^{3}H]CN-IMI and [^{3}H]paroxetine observed in our experiment in the human hippocampus compares well with the binding pattern of [^{3}H]citalopram, and differs from that of [^{3}H]imipramine. Thus, we found low densities in the pyramidal layer of all hippocampal fields but higher labeling in the stratum lacunosum-moleculare. In CA3 and partially CA2, intense labeling was also observed in the stratum radiatum. The same distribution pattern was characteristic for [3H]citalopram, whereas [^{3}H]imipramine heavily labeled pyramidal cells (Duncan et al. 1992). In the human hypothalamus we found more widespread labeling of the hypothalamus nuclei than described in the work of Duncan et al. (1992) for [^{3}H]citalopram. However, these findings are difficult to compare, because no absolute values are presented by Duncan et al. (1992). Only relative comparisons of the binding densities between the nuclei of the hypothalamus and amygdala are given. Other issues have been raised regarding the use of [^{3}H]imipramine to provide an accurate estimation of the 5-HT transporter sites. Our results show, in contrast, that the two selective ligands [^{3}H]CN-IMI and [^{3}H]paroxetine have very similar binding patterns in all human brain structures examined.

Both ligands used in the study are potent antidepressants. Cyanoimipramine is a classic tricyclic compound, whereas paroxetine belongs to a new group of antidepressants—selective 5-HT reuptake inhibitors. Paroxetine has been shown to be at least equally efficacious as an antidepressant as imipramine (Dunbar et al. 1991). The similar binding profiles of the two drugs suggest

.001 according to post hoc Fisher's protected LSD test. The differences shown on the graphs refer to the specific regions within each subdivision (*the names shown enclosed in boxes*): 1-m in the hippocampal formation, SNc in the midbrain, SO in the hypothalamus, NPF in the thalamus, and NAC in the basal ganglia. No significant differences were seen among regions of amygdala. Abbreviations: DG, GL = dentate gyrus granular layer; DG, PL = dentate gyrus, polymorphic layer; CA1, CA2, and CA3 = CA1, CA2, and CA3 fields of the hippocampus; 1-m = stratum lacunosum-moleculare of the hippocampus; SUB = subiculum; PRS = presubiculum; PAS = parasubiculum; ENC = entorhinal cortex; PRC = perirhinal cortex; RN = red nucleus; SNc = substantia nigra pars compacta; SNr = substantia nigra pars reticulata; SNl = Substantia nigra pars lateralis; PN = paranigral nucleus; PBP = parabrachial pigmented nucleus; CLi = caudal linear nucleus of the raphe; RLi = rostral linear nucleus; CG = central gray; NL = lateral amygdaloid nucleus; NBL = nucleus basolateralis of the amygdala; NBM = nucleus basomedialis of the amygdala; AAA = anterior amygdaloid area; NC = nucleus centralis of the amygdala; VCAN = ventral cortical amygdaloid nucleus; APir = amygdalopiriform transition area; SO = supraoptic nucleus of the hypothalamus; DMN = dorsomedial nucleus of the hypothalamus; PV = Paraventricular nucleus of the hypothalamus; LHA = Lateral hypothalamic area; MB = mammillary bodies; TMN = tuberomammillary nucleus of the hypothalamus; LTN = lateral tuberal nucleus of the hypothalamus; DHA = dorsal hypothalamic area; PVN = periventricular nucleus of the hypothalamus; NAV = nucleus anteroventralis of the thalamus; NMD = nucleus medialis dorsalis of the thalamus; NCM = nucleus centrum medianum of the thalamus; NPF = nucleus parafascicularis of the thalamus; NLP = nucleus lateralis posterior of the thalamus; NVPL = nucleus ventralis posterolateralis of the thalamus; NVPM = nucleus ventralis posteromedialis of the thalamus; ZI = zona inserta; DCN = rostral caudate nucleus dorsal part; VCN = rostral caudate nucleus ventral part; DPu = rostral putamen dorsal part; VPu = rostral putamen ventral part; NAC = nucleus accumbens; CCN = caudal caudate nucleus; CPu = caudal putamen; GPe = globus pallidus external part; GPi = globua pallidus internal part; Cl = claustrum; NBM = nucleus basalis of Meynert; BNST = bed nucleus of the stria terminalis; NDB = nucleus of the diagonal band of Broca, vertical division.

that they are likely to target the same protein (5-HT transporter) in the brain structures and, thus, to trigger similar neurochemical responses. The overall good correlation between the [^{3}H]paroxetine and [^{3}H]CN-IMI binding and the density of serotonergic innervation suggests that the brain areas that receive rich serotonergic input such as the hypothalamus and basal forebrain might be common targets of antidepressant drugs. It appears that the density of the 5-HT transporter sites in the brain is not altered by chronic antidepressant treatment. Recent studies with [3H]paroxetine and [^{3}H]CN-IMI as radioligands failed to show changes in their density after chronic treatment with different types of antidepressants, including selective 5-HT transporter inhibitors (Kovachich et al. 1992; Cheetham et al. 1993), or with modifications in thyroid status (Tejani-Butt et al. 1993). At the same time fluoxetine has been shown to upregulate 5-HT transporter sites labeled with [^{3}H]paroxetine in several brain regions (Hrdina and Vu 1993). This may suggest that compounds that do not produce adaptive changes in the density of 5-HT transporter would retain their effectiveness. The etiology of depression and molecular and anatomic targets for antidepressants in the human brain are yet to be understood. Systematic studies of the chemical neuroanatomy of the human brain can ultimately provide insight into neurochemical mechanisms of depression and antidepressant action.

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