Neurotransmitter Receptor and Transporter Binding Profile of Antidepressants and Their Metabolites

MICHAEL J. OWENS, W. NEAL MORGAN, SUSAN J. PLOTT and CHARLES B. NEMEROFF

Laboratory of Neuropsychopharmacology, Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, Atlanta, Georgia

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ABSTRACT

Several new antidepressants that inhibit the serotonin (SERT) and norepinephrine transporters (NET) have been introduced into clinical practice in the past several years. This report focuses on the further pharmacologic characterization of nefazodone and its metabolites within the serotonergic and noradrenergic systems, in comparison with other antidepressants. By use of radioligand binding assays, we measured the affinity (K_i) of 13 antidepressants and 6 metabolites for the rat and human SERT and NET. The K_i values for eight of the antidepressants and three metabolites were also determined for the rat 5-HT1A, 5-HT2A and muscarinic cholinergic receptors, the guinea pig histamine1, receptor and the human alpha1 and alpha2 receptors. These data are useful for predicting side effect profiles and the potential for pharmacodynamic drug-drug interactions of antidepressants. Of particular interest were the findings that paroxetine, generally thought of as a selective SERT antagonist, possesses moderately high affinity for the NET and that venlafaxine, which has been described as a “dual uptake inhibitor”, possesses weak affinity for the NET. We observed significant correlations in SERT (r = 0.965) or NET (r = 0.983) affinity between rat and human transporters. Significant correlations were also observed between muscarinic cholinergic and NET affinity. There are several significant correlations between affinities for the 5-HT1A, 5-HT2A, histamine1, alpha1 and alpha2 receptors. These novel findings, not widely described previously, suggest that many of the individual drugs studied in these experiments possess some structural characteristic that determines affinity for several G protein-coupled, but not muscarinic, receptors.

Nefazodone and venlafaxine are two of several newer antidepressants that have been introduced in the United States in the past several years. These drugs and their metabolites, like the TCAs and SSRIs, are both antagonists of monoamine transporters and receptors in the CNS. The potency of transporter antagonism and receptor binding can theoretically predict both clinical efficacy and side effect profile. With radioligand binding assays, the potency of a given drug for a specific receptor or transporter can be calculated by obtaining the equilibrium inhibition constant (K_i). This constant, unlike IC50 calculations which have been performed in numerous receptor binding studies, is independent of the specific radioligand used or the concentration of radioligand in the assay. This allows for comparison of K_i values across laboratories.

Nefazodone has a chemical structure (fig. 1) seemingly unrelated to SSRIs, TCAs, tetracyclics, bupropion or monoamine oxidase inhibitors. Nefazodone is effective in the treatment of depression, and it has a more favorable side effect profile than the structurally similar antidepressant trazodone (Fontaine et al., 1994; Rickels et al., 1994; Taylor et al., 1995; Robinson et al., 1996). Nefazodone has three major active metabolites (Mayol et al., 1994): hydroxynefazodone, mCPP and a triazoledione tautomer of desethylhydroxynefazodone hereafter termed triazoledione. Venlafaxine, another effective antidepressant, is marketed as a dual serotonin and norepinephrine uptake inhibitor. Its major metabolite is O-desmethylvenlafaxine.

In the present study, we have determined the K_i for 19 commonly used antidepressants or their metabolites for the rat and human SERT and NET. Eleven of these compounds were tested further to determine their affinity for the rat 5-HT1A, 5-HT2A, and muscarinic cholinergic receptors as well as for the guinea pig histamine1 (H1) receptor and the human alpha1 and alpha2 receptors. These studies build upon the seminal studies of Richelson and colleagues (Richelson and Nelson, 1984; Bolden-Watson, 1993; Cusack et al., 1994) by examining the most up-to-date series of antidepressants and their metabolites that target the monoamine transporters.

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ABBREVIATIONS: SERT, serotonin transporter; NET, norepinephrine transporter; 5-HT, 5-hydroxytryptamine; mCPP, meta-chlorophenylpiperazine; AUC, area under the curve; HPLC, high-pressure liquid chromatography; SSRI, serotonin selective reuptake inhibitor; CNS, central nervous system; TCA, tricyclic antidepressant.
Moreover, their affinity at both the rat and human variants of the SERT and NET are compared.

**Methods**

**Tissue sources.** These studies were conducted in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Male Sprague-Dawley rats or guinea pigs were housed with food and water available *ad libitum* in an environmentally controlled animal facility. Animals were sacrificed by guillotine decapitation without anesthesia as approved by the Emory University Animal Use and Care Committee.

For the SERT, NET, 5-HT2A and muscarinic cholinergic binding studies, pooled rat frontal cortex (anterior to the hippocampus) was collected and stored at −280°C until needed. Similarly stored rat hippocampus was used for the 5-HT1A receptor assay. Human frontal and parietal cortex was pooled from six normal control brains obtained from the brain bank of the Emory University Alzheimer’s Disease Research Center for use in the alpha-1 and alpha-2 receptor assays. Postmortem delay in these samples ranged from 6 to 11 hours, and none of the patients were treated with any medications at the time of death that are known to interact with alpha adrenergic receptors.

Samples were homogenized with a Polytron PT 3000 (Brinkmann, 20,000 rpm × 12 seconds) in 30 volumes of their individual assay buffers (table 1) at 4°C, and centrifuged at 43,000 × g for 10 min. The supernatants were decanted and resuspended in 30 volumes of buffer, homogenized, separated into several individual aliquots and centrifuged. For membrane pellets that were used in the 5-HT2A, 5-HT1A, alpha-1 and alpha-2 binding assays, the pellets following the second centrifugation were resuspended in 30 volumes of buffer and the suspensions were preincubated in an oscillating water bath at 37°C for 10 min. After preincubation, these suspensions were recentrifuged at 43,000 × g for 10 min, the supernatants decanted and resuspended in 30 volumes of cold buffer, homogenized, separated into several individual aliquots and centrifuged. The resulting pellets were stored at −70°C until assayed.

Stable transfection of human SERT (Qian et al., 1997) or human NET cDNA (Galli et al., 1995) into HEK-293 (human embryonic kidney) cells has resulted in cell lines exhibiting high-affinity, Na⁺-dependent transport of serotonin or norepinephrine with pharmacological properties identical with those of native membranes. These have been provided to us by Randy Blakely, Ph.D. (Vanderbilt University). Both cell lines were grown in ten tray Nunc cell factories (6,320 cm²) to confluence in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum supplemented with L-glutamine (2 mmol/l final concentration), penicillin (100 μg/ml) and 100 units/ml streptomycin in a humidified incubator at 37°C containing 5% CO₂. The selecting antibiotic geneticin sulfate (250 μg/ml) was used during all growth phases. Membranes were harvested using

![Fig. 1. Chemical structures of the antidepressants and metabolites used in the studies.](image-url)
used a single concentration of either [3H]serotonin (final concentration 20 nmol/l; 5 nmol/l [3H]serotonin, 15 nmol/l serotonin) or [3H]norepinephrine (final concentration 20 nmol/l; 5 nmol/l [3H]norepinephrine, 15 nmol/l norepinephrine).

Assay conditions for binding and transport assays

| Receptor/Transporter | Ligand | Membrane Source (µg protein/tube) | Incubation Conditions | Final Volume
|----------------------|--------|----------------------------------|-----------------------|--------------
| SERT                 | [3H]Citalopram | Rat cortex (150 µg) | 52.6 mM Tris-HCl, 126.4 mM NaCl, 5.26 mM KCl, pH 7.9, 22°C, 1 hr | 2 ml
| hSERT               | [3H]Citalopram | HEK-293/hSERT cDNA (35 µg) | 52.6 mM Tris-HCl, 126.4 mM NaCl, 5.26 mM KCl, pH 7.9, 22°C, 1 hr | 2 ml
| hSERT               | [3H]Serotonin | HEK-293/hSERT cDNA | Krebs-Ringer Henseleit buffer, 105 µM ascorbate, 105 µM pargyline, pH 7.4, 37°C, 10 min | 1 ml
| NET                 | [3H]Nisoxetine | Rat cortex (175 µg) | 52.6 mM Tris-HCl, 316 mM NaCl, 5.26 mM KCl, pH 7.4, 4°C, 4 hr | 1 ml
| hNET                | [3H]Nisoxetine | HEK-293/hNET cDNA (75 µg) | 52.6 mM Tris-HCl, 316 mM NaCl, 5.26 mM KCl, pH 7.4, 4°C, 4 hr | 1 ml
| hNET                | [3H]Norepinephrine | HEK-293/hNET cDNA | Krebs-Ringer Henseleit buffer, 105 µM ascorbate, 105 µM pargyline, pH 7.4, 37°C, 10 min | 1 ml
| 5-HT1A              | [3H]-OH-DPAT | Rat hippocampus (175 µg) | 52.6 mM Tris-HCl, 1.05 mM MnCl2, pH 7.7, 22°C, 1 hr | 2 ml
| 5-HT2A              | [3H]Ketanserin | Rat cortex (175 µg) | 52.6 mM Tris-HCl, 4.2 mM CaCl2, 30 nM prazosin, pH 7.4, 37°C, 1 hr | 1 ml
| alpha-1             | [3H]Prazosin | Human cortex (125 µg) | 52.6 mM Tris-HCl, pH 7.5, 22°C, 1 hr | 5 ml
| alpha-2             | [3H]Prazosin | [O-methyl-3H]Rauwolscine | Human cortex (200 µg) | 52.6 mM Tris-HCl, pH 7.4, 22°C, 1 hr | 2 ml
| Histamine (H1)      | [3H]Pyrilamine | Guinea pig whole brain (400 µg) | 52.6 mM K2HPO4, 52.6 mM NaCl, pH 7.5, 22°C, 1 hr | 5 ml
| Muscarine           | [3H]-N-methylscopolamine | Rat cortex (25 µg) | 77.4 mM Na2HPO4, 22.6 mM NaH2PO4, 5.27 mM MgCl2, pH 7.4, 22°C, 1 hr | 5 ml

* Assay buffer composition, temperature and length of incubation.
* All competing drugs added as 1/20th the final volume in 5 mM HCl.

37°C phosphate-buffered saline containing 0.53 mM/l ethylenediaminetetraacetic acid, separated into aliquots, and centrifuged at 2000 × g for 10 min. The supernatants were decanted and the pellets homogenized as described above.

General radioligand binding assay methods. For all data shown in the manuscript, serial dilution of radioligands or competing drugs was carried out in borosilicate glass tubes silanized with Prosil 28 (PCR Inc., Gainesville FL). Fresh competing drug was weighed out for each individual competition curve. All competing drugs were initially dissolved in 50% ethanol containing 5 mM HCl at a drug concentration of 1 mg/ml. Subsequent serial dilutions were performed in silanized glass tubes in 5 mM HCl and added as 1/20th the final total volume of the assay tubes. This did not alter the pH of any of the buffer systems. We compared the use of silanized glass tubes with polystyrene and polypropylene tubes and found that silanized glass tubes were preferable for preparing serial dilutions (M. J. Owens and W. N. Morgan, unpublished observations). The total incubation volumes and membrane protein concentrations of all assays were adjusted such that the free ligand concentration was at least 95% of the total ligand concentration (see table 1). For all membrane binding assays, with the exception of those using human brain tissue which we were not able to study, we observed that the Kd values of freshly prepared tissue pellets and previously frozen tissue pellets are identical, although a 0 to 8% decrease in Bmax was observed among the various assays (M. J. Owens and W. N. Morgan, unpublished observations). Competition assays used 19 to 20 concentrations of competing ligand in triplicate over a maximum concentration range of 10^-13 to 10^-4.6 mol/l. The chosen concentrations of competing ligand were adjusted for each assay to provide at least 10 points on the curve between 10% and 90% displacement. The only exceptions were the transport and radioligand binding assays with the hSERT and hNET cell lines which used 12 concentrations of competing ligand. All competition binding assays used a single concentration of [3H]labeled radioligand equal to the calculated Kd of that ligand for its receptor (table 2). Competitive transport assays used a single concentration of either [3H]serotonin (final concentration 20 nmol/l; 5 nmol/l [3H]serotonin, 15 nmol/l serotonin) or [3H]norepinephrine (final concentration 20 nmol/l; 5 nmol/l [3H]norepinephrine, 15 nmol/l (-)-norepinephrine).

For each different receptor assay, the results of six separate saturation studies were simultaneously analyzed by use of the computer program LIGAND (Munson and Rodbard, 1980). In competition assays, the results of at least three separate competition assays were analyzed by use of the computer program PRISM 2.0 (GraphPad Software, Inc., San Diego, CA). In all instances, LIGAND or PRISM analysis revealed that the data were best fit by a one-site model rather than a two-site model. All Kd data are expressed as geometric mean ± S.E. in nanomoles per liter. Geometric means and S.E. were calculated by the method described by De Lean et al. (1982). pKd correlations were conducted by Pearson correlations with SAS software ( Cary, NC).
TABLE 2
Affinity of the radioligands and their $B_{\text{max}}/V_{\text{max}}$ in the tissues used in these studies

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Receptor</th>
<th>Concentration Range</th>
<th>Specific Binding$^a$</th>
<th>$K_i/K_d$</th>
<th>$B_{\text{max}}/V_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^3]$H Citalopram</td>
<td>Rat SERT</td>
<td>0.05–2.50</td>
<td>89%; 1 µmol/l chlorimipramine</td>
<td>0.56</td>
<td>357</td>
</tr>
<tr>
<td>$[^3]$H Citalopram</td>
<td>hSERT</td>
<td>0.02–2.50</td>
<td>85%; 1 µmol/l chlorimipramine</td>
<td>1.06</td>
<td>1575</td>
</tr>
<tr>
<td>$[^3]$H Serotonin</td>
<td>hSERT</td>
<td>40–6000</td>
<td>95%; 500 nmol/l paroxetine</td>
<td>533</td>
<td>$4.10 \times 10^{-11}$ mol/cell/min</td>
</tr>
<tr>
<td>$[^3]$H Nisoxetine</td>
<td>Rat NET</td>
<td>0.07–5.50</td>
<td>84%; 1 µmol/l mazindol</td>
<td>0.73</td>
<td>194</td>
</tr>
<tr>
<td>$[^3]$H Nisoxetine</td>
<td>hNET</td>
<td>0.05–5.0</td>
<td>87%; 1 µmol/l mazindol</td>
<td>1.24</td>
<td>2531</td>
</tr>
<tr>
<td>$[^3]$H Norepinephrine</td>
<td>hNET</td>
<td>40–6000</td>
<td>95%; 1 µmol/l mazindol</td>
<td>1608</td>
<td>$1.73 \times 10^{-16}$ mol/cell/min</td>
</tr>
<tr>
<td>$[^3]$H OH-DPAT</td>
<td>Rat 5-HT$_{1A}$</td>
<td>0.025–2.0</td>
<td>94%; 10 µmol/l 5-HT</td>
<td>0.13</td>
<td>187</td>
</tr>
<tr>
<td>$[^3]$H Ketanserin</td>
<td>Rat 5-HT$_{1A}$</td>
<td>0.015–10.0</td>
<td>80%; 1 µmol/l cinanserin</td>
<td>0.77</td>
<td>233</td>
</tr>
<tr>
<td>$[^3]$H N-methylscopolamine</td>
<td>Rat muscarinic</td>
<td>0.005–0.6</td>
<td>98%; 250 nmol/l atropine</td>
<td>0.036</td>
<td>994</td>
</tr>
<tr>
<td>$[^3]$H Pyrilamine</td>
<td>Guinea pig H$_{1}$</td>
<td>0.075–3.0</td>
<td>97%; 1 µmol/l chlorpheniramine</td>
<td>0.15</td>
<td>137</td>
</tr>
<tr>
<td>$[^3]$H Prazosin</td>
<td>Human alpha-1</td>
<td>0.004–2.50</td>
<td>89%; 10 µmol/l phenolamine</td>
<td>0.037</td>
<td>88</td>
</tr>
<tr>
<td>$[^3]$O-methyl-$[^3]$H Rauwolscine</td>
<td>Human alpha-2</td>
<td>0.15–5.0</td>
<td>75%; 1 µmol/l yohimbine</td>
<td>0.86</td>
<td>184</td>
</tr>
</tbody>
</table>

$^a$ Data presented as % specific binding as a function of total binding in competition curves (fig. 2A to L); final concentration of drug used to defined specific binding/transport.

**Results**

Affinity of the radioligands and their $B_{\text{max}}/V_{\text{max}}$ in the tissues used in these studies. Table 2 shows the results of the saturation analyses for the radioligands used in the present studies, which served to determine radioligand and tissue concentrations necessary for the competition studies. Calculation of $K_i$ values required determination of radioligand $K_a$ values in this laboratory as values reported in the literature under similar assay conditions are not always identical. The specific assay methods were based on those we have previously used successfully in this laboratory or were based on those reported in the literature. Specifically, $[^3]$H OH-DPAT binding was performed as described and characterized by Hall et al. (1985, 1986); $[^3]$H ketanserin binding was performed as described in McKenna et al. (1989) and Owens et al. (1991); and $[^3]$H N-methylscopolamine binding was modified from the binding described by Dörje et al. (1991). $[^3]$H Prazosin binding used human cortical tissue because prazosin showed affinity for both alpha-1 and alpha-2 receptors in rat cortex, unlike human cortex in which prazosin is selective for the alpha-1 receptor (Cheung et al., 1982). [O-methyl-$[^3]$H]rauwolscine binding was also performed in human brain because yohimbine and rauwolscine, two alpha-2 antagonists, have significantly different affinities in rat versus human cortex (Summers et al., 1983; Cheung et al., 1982; Ruffolo et al., 1991). $[^3]$H Pyrilamine binding to H$_1$-histamine receptors was performed in guinea pig brain tissue because the pharmacological profile of pyrilamine in this species more closely resembles that observed with the human H$_1$ receptor than does binding in rat brain tissue (Chang et al., 1979; Hill and Young, 1980; Hill, 1990).

Affinities of antidepressants and their metabolites. Table 3 lists the affinities ($K_i$) of the various antidepressants and their metabolites for the transporters and receptors examined in the present studies. The averaged competition curves for each transporter/receptor system are shown in figure 2, A to L. The data for the SERT and NET in table 3 were used to determine relative selectivity among the various drugs for the two transporters (fig. 3). Dividing the $K_i$ for the NET by the $K_i$ for the SERT yields a unitless number where 1 equals no selectivity (i.e., equal affinity for both transporters). Values $>1$ represent greater SERT selectivity. Values $<1$ represent greater NET selectivity.

Correlation of affinities between receptors and species. Table 4 lists the significant correlations observed comparing affinity at one transporter/receptor and affinity at another. Figure 4, A to D, compares the affinities with use of

**Drugs.** $[^3]$H Citalopram (3012 GBq/mmol), $[^3]$H 8-OH-2-di (dipropylamo)tetral (4914 GBq/mmol), $[^3]$H ketanserin (2290 GBq/mmol), $[^3]$H prazosin (2886 GBq/mmol) and $[^3]$H pyrilamine (866 GBq/mmol) were obtained from New England Nuclear (Boston MA). $[^3]$H Serotonin (4084 GBq/mmol), $[^3]$H nisoxetine (3105 GBq/mmol), $[^3]$H noradrenaline (2741 GBq/mmol) and $[^3]$H N-methylscopolamine (855 GBq/mmol) were obtained from Amersham Inc. (Buckinghamshire UK). Nefazodone, hydroxynefazodone, mCPP, trazodone and triazolodione were gifts from Bristol Myers-Squibb (Wallington, CT). Fluoxetine, nortriptyline, and nortriptylamine were gifts from the Eli Lilly and Co. (Indianapolis, IN). Venlafaxine and O-desmethylvenlafaxine were gifts from Wyeth-Ayerst Pharmaceuticals (Princeton, NJ). Paroxetine was a gift from SmithKline Beecham Pharmaceuticals (West Sussex, England). Fluvoxamine was a gift of Solvay Pharmaceuticals (Marietta, GA). Citalopram was a gift from H. Lundbeck A/S (Copenhagen-Valley, Denmark). Sertraline and desmethylsertraline were gifts from Pfizer Pharmaceuticals (Groton, CT). Imipramine and desipramine were purchased from Sigma (St. Louis, MO). Amtriptyline, atropine, chloroimipramine, chlorpheniramine, cinanserin, mazindol, phenolamine, serotonin HCl and yohimbine were purchased from Research Biochemicals Inc. (Natick, MA).

Measurement of nefazodone concentrations in serial dilutions from competition assays. To determine why serial dilutions prepared in 5 mmol/l HCl or individual serial dilutions prepared in silanized glass tubes. Nefazodone concentrations in serial dilutions prepared in 5 mmol/l HCl or assay buffer from the $[^3]$H citalopram binding experiments were measured by HPLC with a modification of the method of Franc et al. (1991). Of the various individual dilutions, 20 to 100 µl were injected onto a 150 × 4.6 mm BDS-Hypersil-Phenyl 5-µm column with guard (Keystone Scientific Inc., Bellefonte, PA) with a mobile phase consisting of 20 mmol/l NH$_4$C$_6$H$_5$O$_7$ buffer (pH = 3.0), methanol and acetonitrile at a ratio of 53:15:32 at a flow rate of 1.0 ml/min. Peaks were identified by an LDC (Riviera Beach, FL) ultraviolet detector set at 250 nm. Sensitivity of the assay was 2.5 ng.

Serial dilutions were prepared fresh every 60 min to ensure that samples sitting on the bench were not degraded/altered. As in the binding experiments, peak heights of serial dilutions in 5 mmol/l HCl represented 100% recovery. Samples from identical serial concentrations from both duplicate conditions were injected before measurement of the next concentration. The order of samples (i.e., 5 mmol/l HCL in assay buffer) were randomized for each concentration.
the same ligand ($^{3}$H)citalopram or $^{3}$H)nisoxetine) to bind to the rat and human SERT and NET, respectively, or the affinities calculated with the radioligands above versus active transport of $^{3}$H]5-HT or $^{3}$H]NE. As expected, highly significant correlations were found between the affinity of the antidepressants for the transporters measured by $^{3}$H)citalopram or $^{3}$H)nisoxetine and active monoamine transport in cultured cells (fig. 4, B and D). As shown in figure 4, A and C, competition assays with either $^{3}$H)citalopram or $^{3}$H)nisoxetine showed highly significant correla-
tions (P < .0001) comparing the rat and human versions of the respective transporters. Indeed, linear regression yielded almost a perfect one-to-one correlation for both the SERT and NET. However, in the SERT, the TCAs amitriptyline, nor-
triptyline, imipramine, desipramine and chloroimipramine were 4.5 to 10 times more potent (table 3) at the human SERT. This increased potency is shown by the TCAs being below the regression line in figure 4A.

As shown in table 4, several correlations were observed in the affinities between different receptor/transporter systems. The potency of the various antidepressants for either the SERT or NET was positively correlated with the affinity at
rat muscarinic receptors. Positive correlations between the affinities for the 5-HT$_{2A}$, 5-HT$_{1A}$, H$_1$, alpha-1 and alpha-2 receptors were also observed. The strongest correlations were observed between alpha-1 and alpha-2 receptors ($r = 0.920$) and 5-HT$_{1A}$ and alpha-2 receptors ($r = 0.933$). The results suggest that the antidepressants tested here possess certain individual structural features that render a certain degree of potency to a variety of G protein-coupled receptors, the exception being muscarinic receptors. Finally, if not for triazolodione which possesses negligible SERT activity and venlafaxine which is inactive at the 5-HT$_{2A}$ and 5-HT$_{1A}$ receptors, a negative correlation would be observed between SERT affinity and 5-HT$_{2A}$ or 5-HT$_{1A}$ affinity ($P < .01$, data not shown).

As shown in Table 5, highly significant correlations between the affinity of the antidepressants for rodent and human versions of the 5-HT$_{1A}$, 5-HT$_{2A}$, H$_1$ and muscarinic receptors were observed. Although the affinities were highly correlated between species, we observed that the absolute potencies were higher in our studies with rat or guinea pig brain. However, a similar higher affinity for all drugs was observed in our studies with the alpha-1 and alpha-2 receptors which used human cortex. We believe this could be the result of our using 5 mmol/l HCl to prepare our serial dilu-
tions of competing ligand, which does not allow loss of competing drug as observed when serial dilutions are prepared in assay buffer (see below). Additionally, differences in assay conditions could contribute to the differences, but this should be minimal when $K_i$ or $K_d$ values are calculated rather than IC$_{50}$ values. Perhaps more likely are species differences that result in different absolute affinities, but do not change rank orders of potency. With a limited amount of human tissue available, we did note that the phenylpiperazine antidepressants (nefazodone, its metabolites and trazodone), but not the other antidepressants examined, were 5- to 10-fold less potent at the human H$_1$ than the guinea pig H$_1$ receptor (data not shown). However, these values were still substantially more potent for nefazodone than those reported by Cusack et al. (1994).

**HPLC analysis of nefazodone concentrations in serial dilutions.** Serial dilutions of competing drugs are routinely prepared in assay buffer after dissolution in the appropriate solvent. We observed that after complete dissolution of the various drugs in an acid/ethanol mixture and further serial dilution in assay buffer, several drugs produced competition curves with very high Hill coefficients ($n_{H} > 1.8$) and could not be accurately curve fitted (fig. 5) (Morgan et al., 1995). These very steep curves were observed...
in rat SERT, 5-HT\textsubscript{2A} and 5-HT\textsubscript{1A} receptor assays (they were not examined in the other assays) and were most pronounced for nefazodone, hydroxynefazodone, trazodone, sertraline and amitriptyline (data not shown). They were more modestly observed for triazoledione and paroxetine, and not observed at all for mCPP, venlafaxine, fluoxetine and desipramine. In addition to the very steep competition curves produced by certain drugs, the drugs also appeared substantially less potent as shown by IC\textsubscript{50} values. (K\textsubscript{i} could not be calculated in the steep curves because of the poor curve fit.)

To compare the absolute amount of nefazodone in several types of serial dilutions, we prepared fresh serial dilutions of nefazodone in: 5 mmol/l HCl as was used in all data presented in table 3 and figure 2, A to L, and in buffer from the \textsuperscript{3}Hcitalopram assay. Samples were immediately subjected to HPLC analysis and compared for nefazodone concentrations (table 6). Serial dilution of nefazodone prepared in 5 mmol/l HCl was equal to that prepared in mobile phase (M. Owens, unpublished observations) and was taken to equal 100% recovery. As shown in table 6, dilutions prepared in assay buffer were significantly lower. Moreover, recovery decreased with increasing dilution. The addition of H\textsuperscript{+} (25 μl of 1 mol/l HCl) to the assay buffer increased recovery to a limited extent (data not shown). Using the actual nefazodone concentrations as determined by HPLC (table 6), we compared the \textsuperscript{3}Hcitalopram competition curves in rat cortex
produced by nefazodone in 5 mmol/l HCl assay buffer, and assay buffer corrected for actual nefazodone concentrations (fig. 6). As shown in figure 6, the corrected nefazodone curve was now similar to the nefazodone in HCl curve in terms of the observed potency of nefazodone. Moreover, the curve could now be fit to an idealized one-site competition model and had a Hill coefficient near 1.0.

**Discussion**

Nefazodone, venlafaxine, fluvoxamine and mirtazapine have all recently been introduced in the United States for clinical use. These compounds are inhibitors of monoamine transporters, with the exception of mirtazapine, which appears to be primarily an alpha-2 antagonist at auto- and heteroreceptors as well as a potent 5-HT2A and 5-HT3 antagonist (de Boer and Ruigt, 1995). Unlike venlafaxine and fluvoxamine, which only have high affinity for monoamine transporters, nefazodone possesses potent 5-HT2A receptor antagonism as well as monoamine transporter antagonist properties (Taylor et al., 1995; Owens et al., 1995). Several years have passed since the comprehensive studies of Richelson and colleagues (Bolden-Watson and Richelson, 1993; Cusack et al., 1994) appeared in which the receptor binding profile of several antidepressants and metabolites were examined. Many earlier studies either focused on a single re-
ceptor/transporter or did not include active metabolites for many of the compounds. Moreover, many studies reported IC_{50} inhibition values which highly depended on both the radioligand used and assay conditions, and were difficult to compare across laboratories. Thus, we examined in detail the binding profile of several antidepressants and their metabolites that are monoamine transporter antagonists with particular attention paid to nefazodone (fig. 1).

Antagonism/inhibition of the SERT was established by competition for \[^{3}H\text{]citalopram}\ from either rat frontal cortical membranes or from a HEK-293 cell line stably transfected with the human SERT or antagonism of \[^{3}H\text{]5-HT}\ transport into intact HEK-293 cells expressing the human SERT. \[^{3}H\text{]Citalopram}\ possesses several advantages compared with other ligands for labeling the SERT including the greatest selectivity, higher specific activity and an affinity which, unlike \[^{3}H\text{]paroxetine}\, provides sufficient signal without depleting free ligand concentration at typical assay volumes and protein amounts (D’Amato et al., 1987; Owens et al., 1996). Although it is not known for certain, citalopram and paroxetine probably label the site near or at the site which serotonin itself occupies for transport (Barker et al., 1994; Barker and Blakely, 1996). Nevertheless, actual inhibition of \[^{3}H\text{]monoamine}\ transport may represent the best measure of transporter antagonism.

As shown in table 3 and figure 2, A to C, only the nefaz-
odone metabolite, triazoledione, did not possess any affinity for the SERT. Only moderate affinity for the SERT was observed for nefazodone and its metabolites. Previously, both nefazodone and hydroxynefazodone have demonstrated $K_i$ values between 137 and 181 nmol/l for inhibition of rat $[3H]$5-HT transport (Bolden-Watson and Richelson, 1993; Taylor et al., 1995). Moreover, serum levels observed in rats which significantly, but not completely, inhibit 5-HT transport are consistent with those obtained with oral nefazodone dosages of 300 to 500 mg/day in humans which demonstrated clinical antidepressant efficacy (Owens et al., 1995; Robinson et al., 1996).

The potency of the various antidepressants at the rat SERT agrees very well with those of other studies which typically included only a few compounds (D’Amato et al., 1987; Plenge and Mellerup, 1991; Cheetham et al., 1993). Unlike, sertraline, amitriptyline and imipramine, the desmethyl metabolites of fluoxetine and venlafaxine had potency similar to their parent compounds. Desmethylsertraline still retains high potency and accumulates to 1.6- to 2.1-fold higher levels than sertraline in plasma, but in vivo data suggest that it may not contribute significantly to inhibition of 5-HT transport clinically (Sprouse et al., 1996). This conclusion may be born out in the finding of significantly reduced potency versus sertraline in 5-HT transport via the human SERT.

The affinity of the various compounds for displacing $[3H]$citalopram from the rat and human versions of the SERT were very similar and highly correlated (tables 3 and 4, fig. 4A). The exceptions are the clearly higher potencies of the TCAs amitriptyline, nortriptyline, imipramine, desipramine and chloroimipramine for the human SERT. The increased potency of the tricyclics at the human SERT compared with the rat SERT agrees very well with the findings of Barker and colleagues (Barker et al., 1994; Barker and Blakely, 1996) who used chimeric rat and human SERTs. This property of the tricyclics appears to be attributed to a region near putative transmembrane domain 12 of the human SERT which imparts some species (human) preference for TCAs. Inhibition of $[3H]$5-HT transport was also highly correlated with the ability of individual compounds to displace $[3H]$citalopram (tables 3 and 4, fig. 4B). These results agree very well with those of Bolden-Watson and Richelson (1993) and Cheetham et al. (1993).

$[3H]$Nisoxetine was used to label rat and human NETs. Nisoxetine is 400- and 1000-fold more potent in binding to the NET than the DAT and SERT, respectively. The binding is saturable and Na$^+$-dependent to a single class of binding sites (Tejani-Butt, 1992). As shown in table 3 and figure 2, D and E, other than the TCAs, paroxetine was the only other compound possessing moderately high affinity. Even in the face of its high relative SERT selectivity (fig. 3), preliminary studies with serum concentrations of paroxetine similar to those used to treat panic disorder antagonize the NET in rats (M. J. Owens, D. L. Knight and C. B. Nemeroff, unpublished observations). Although nefazodone and trazodone possess


### TABLE 4

<table>
<thead>
<tr>
<th>Correlation Coefficient (r)</th>
<th>P value</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat SERT × hSERT [3H]citalopram</td>
<td>0.964</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>hSERT [3H]5-HT × hSERT [3H]citalopram</td>
<td>0.962</td>
<td>&lt;.0011</td>
</tr>
<tr>
<td>hSERT [3H]5-HT × rat SERT</td>
<td>0.961</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Rat NET × hNET [3H]nisoxetine</td>
<td>0.980</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>hNET [3H]NE × hNET [3H]nisoxetine</td>
<td>0.982</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>hNET [3H]NE × rat NET</td>
<td>0.957</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Muscarinic × rat SERT</td>
<td>0.618</td>
<td>.043</td>
</tr>
<tr>
<td>hSERT [3H]citalopram</td>
<td>0.704</td>
<td>.016</td>
</tr>
<tr>
<td>hSERT [3H]5-HT</td>
<td>0.701</td>
<td>.016</td>
</tr>
<tr>
<td>rat NET</td>
<td>0.806</td>
<td>.003</td>
</tr>
<tr>
<td>hNET [3H]nisoxetine</td>
<td>0.820</td>
<td>&lt;.002</td>
</tr>
<tr>
<td>hNET [3H]NE</td>
<td>0.751</td>
<td>.008</td>
</tr>
<tr>
<td>5-HT2A × 5-HT1A</td>
<td>0.847</td>
<td>.001</td>
</tr>
<tr>
<td>H1</td>
<td>0.842</td>
<td>.001</td>
</tr>
<tr>
<td>alpha-1</td>
<td>0.865</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>alpha-2</td>
<td>0.868</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>5-HT1A × 5-HT2A</td>
<td>0.847</td>
<td>.001</td>
</tr>
<tr>
<td>H1</td>
<td>0.639</td>
<td>.034</td>
</tr>
<tr>
<td>alpha-1</td>
<td>0.803</td>
<td>.003</td>
</tr>
<tr>
<td>alpha-2</td>
<td>0.933</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>H1 × 5-HT2A</td>
<td>0.842</td>
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</tr>
<tr>
<td>5-HT1A</td>
<td>0.639</td>
<td>.034</td>
</tr>
<tr>
<td>alpha-1</td>
<td>0.739</td>
<td>.009</td>
</tr>
<tr>
<td>alpha-2</td>
<td>0.613</td>
<td>.046</td>
</tr>
<tr>
<td>Alpha-1 × 5-HT2A</td>
<td>0.865</td>
<td>&lt;.001</td>
</tr>
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<td>0.803</td>
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<tr>
<td>H1</td>
<td>0.739</td>
<td>.009</td>
</tr>
<tr>
<td>alpha-2</td>
<td>0.920</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Alpha-2 × 5-HT2A</td>
<td>0.868</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>5-HT1A</td>
<td>0.933</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>H1</td>
<td>0.613</td>
<td>.046</td>
</tr>
<tr>
<td>alpha-1</td>
<td>0.920</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

* Only those correlations that were significant are shown.

similar SERT affinity, trazodone is inactive at the NET suggesting that important structural requirements for NET activity are found in either the phenoxyethyl substituent or the 5-substituent of the triazole moiety, or both (fig. 1).

Our findings for the inhibition of [3H]NE transport in cells expressing the human NET are very similar to those observed for inhibition of [3H]5-HT transport by the human SERT that in the rank order of potency was similar to that observed for radioligand binding (table 3, figs. 2, D and E, and 4D). Of the same drugs tested, our calculated Ki values were identical with those reported by Pacholczyk et al. (1991) for the transfected human NET.

As mentioned above, absolute affinity was highly correlated among the compounds for the rat and human NET and between the use of [3H]nisoxetine and [3H]NE (table 3, fig. 4, C and D). Indeed, this correlation was even stronger than that observed for the SERTs because of the similar potencies of the TCAs for rat and human NETs. These data suggest that rat tissue does provide useful data regarding the potency of various compounds for the human NET and that this can be reflected in radioligand binding studies which are easier to perform.

The relative selectivities for the SERT and NET are shown in figure 3. The rank order of selectivity varies slightly depending on the assay method. Desipramine and nortriptyline are clearly the most NET selective of the compounds tested, and sertraline and citalopram are the most SERT selective. Although relatively weak antagonists of the SERT and NET, nefazodone and hydroxynefazodone are the closest to being called “dual uptake inhibitors.” Our data show that venlafaxine and O-desmethylvenlafaxine have, at a minimum, a > 15-fold selectivity for the SERT. These selectivities are relative, and with escalating dosages and increased free drug concentrations in serum, even relatively selective drugs can begin to, or fully, bind to other transporters/receptors.

Amitriptyline, nefazodone and hydroxynefazodone were potent at displacing [3H]ketanserin binding from the rat cortical 5-HT2A receptor (table 3). Trazodone also displayed significant potency (Ki = 20 nmol/l). These data agree closely with data reported previously (Wander et al., 1986; Seeman, 1993; Cusack et al., 1994). Our results from rat tissue are highly correlated with those of Cusack et al. (1994) who used human cortex (table 5), although our antidepressants displayed somewhat higher affinity in all instances. Once again, we believe this could be related to the method of serial dilution and/or species differences in absolute affinity.

The phenylpiperazine derivatives possessed moderate affinity for the rat hippocampal 5-HT1A receptor (table 3). mCPP is thought to act as an agonist, whereas it is not known whether the parent drugs are antagonists or agonists, although antagonism is more likely. It has been suggested that combined with the potent 5-HT2A antagonism, these compounds do augment 5-HT1A-mediated function (Taylor et al., 1995). These findings agree with those of Richelson and colleagues (Wander et al., 1986; Cusack et al., 1994) and those reported in Seeman (1993). Our data in rat hippocampus was highly correlated with that in human tissue (table 5).

As previously reported by the manufacturer (Paxil, package insert), paroxetine possesses moderately high affinity for muscarinic receptors similar to that observed for the TCA desipramine (table 3). Once again, our data in rats are highly correlated with data observed in humans (Stanton et al., 1993; Cusack et al., 1994) and in Seeman (1993).

As noted under “Methods,” H1 receptors in guinea pig brain display closer pharmacology to the human H1 receptor than do rat H1 receptors. Amitriptyline was the most potent drug tested. However, trazodone, nefazodone, hydroxynefazodone and triazolodione all displayed moderately high potency (Ki values < 30 nmol/l). These findings are in sharp contrast to those reported by Cusack et al. (1994) who reported Ki values for nefazodone and trazodone as 24,000 and 1,100 nmol/l, respectively. This discrepancy may be explained by our findings of loss of drug accompanying serial dilution of the drugs (see below) and species differences for the binding affinity of these two drugs (see “Results”). Even with these discrepancies, our data in guinea pig brain was highly correlated with the data reported by Cusack et al. (1994) in human cortex (table 5).

Previous work has provided substantial evidence that rat cortical alpha adrenoceptors have pharmacological characteristics that are different from their human counterparts (Ruffolo et al., 1991). Therefore, we used human cortical tissue for our studies of antidepressant alpha-1 and alpha-2 affinities. For each drug tested, we observed that they possessed a higher affinity for alpha-1 receptors than for alpha-2 receptors (table 3). The high potency displayed by nefazodone and hydroxynefazodone is surprising because the former is
reportedly associated with significantly less orthostasis when compared with amitriptyline. Nefazodone and hydroxynefazodone were the only drugs tested that had moderate affinities (\(<100\ \text{nmol/l}\) for the $\alpha_2$ receptor. As expected, because of the use of the same species tissues, both our $\alpha_1$ and $\alpha_2$ data were highly correlated with the data reported by Cusack et al. (1994). However, they were not any greater than that observed with the 5-HT$_{1A}$, 5-HT$_{2A}$, H$_1$, $\alpha_1$-1 and $\alpha_2$-2 receptors suggests that these individual drugs possess some structural characteristics that determine affinity for several G protein-coupled receptors, the only exception being the muscarinic receptors. Standard hydropathy analyses of the primary sequences for these receptors found that the N-terminal region of the rat m$_1$ receptor, which contributes most of the muscarinic binding in rat cortex, possesses...
Nefazodone is an effective antidepressant that is marketed exclusively: amitriptyline, desipramine, nefazodone, fluoxetine, paroxetine, sertraline, trazodone and venlafaxine. Human tissue was used in both studies of \( \alpha \)-2 receptor affinities.

One could speculate that this may allow nefazodone to act, in some manner similar to the new antidepressant mirtazapine on \( \alpha \)-2 auto- and heteroreceptors. Additionally, the moderately high affinity for 5-HT\(_{1A}\) receptors suggests that nefazodone may have some inherent properties that mimic the strategy being utilized by the use of pindolol augmentation as a means to block 5-HT\(_{1A}\) somatodendritic autoreceptors and hasten clinical response and/or convert antidepressant nonresponders to responders. Although trazodone shows a striking similarity to nefazodone \textit{in vitro}, with the exception of lack of NET antagonism, trazodone rarely produces priapism, whereas nefazodone does not. The mechanism of this difference is not known or discernible from the present binding studies.

The TCAs exhibited a binding profile very similar to that reported previously. Thus, amitriptyline, imipramine, nor- triptyline and desipramine showed high affinity for the SERT, particularly the human version, and for the NET in which the secondary amines were more potent. In agreement with these findings, amitriptyline and desipramine showed high affinity for the rat 5-HT\(_{1A}\) receptor, whereas nefazodone did not. However, nefazodone both antagonizes the rat 5-HT\(_{1A}\) receptor and has a binding affinity for the human 5-HT\(_{1A}\) receptor.

**TABLE 5**

<table>
<thead>
<tr>
<th>Correlation Coefficient (r)</th>
<th>P value</th>
<th>n</th>
<th>Linear regression ( y = mx + b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human 5-HT(<em>{2A}) × rat 5-HT(</em>{2A})</td>
<td>0.974</td>
<td>&lt;.0001</td>
<td>8</td>
</tr>
<tr>
<td>Human 5-HT(<em>{2A}) × rat 5-HT(</em>{1A})</td>
<td>0.945</td>
<td>.0004</td>
<td>8</td>
</tr>
<tr>
<td>Human H(<em>{1}) × guinea pig H(</em>{1})</td>
<td>0.854</td>
<td>.0065</td>
<td>8</td>
</tr>
<tr>
<td>Human muscarinic × rat muscarinic</td>
<td>0.992</td>
<td>&lt;.0001</td>
<td>8</td>
</tr>
<tr>
<td>Human alpha-1</td>
<td>0.985</td>
<td>&lt;.0001</td>
<td>8</td>
</tr>
<tr>
<td>Human alpha-2</td>
<td>0.933</td>
<td>.0007</td>
<td>8</td>
</tr>
</tbody>
</table>

\( a \) \( pK_i \) values were compared for the following antidepressants using data from the present study and the study of Cusack \textit{et al.} (1994), which used human tissue exclusively: amitriptyline, desipramine, nefazodone, fluoxetine, paroxetine, sertraline, trazodone and venlafaxine. Human tissue was used in both studies of \( \alpha \)-1 and \( \alpha \)-2 receptor affinities.

\( b \) \( y \) represents \( pK_i \) values obtained in human tissue by Cusack \textit{et al.} (1994); \( x \) represents \( pK_i \) values obtained in the present manuscript. A regression formula of \( y = 1.0x + 0 \) would represent an absolute one-to-one correspondence between the two studies. Slopes < 1 represent that a relatively higher affinity for a given compound was observed in the present study compared with that of Cusack \textit{et al.} (1994).

![Fig. 5. Nefazodone competition curves for the 5-HT\(_{2A}\) receptor in rat cortex. NEFAZODONE in HCl is the final averaged curve taken from figure 2H \( (K_i = 7.1 \text{ nmol/l}) \). NEFAZODONE in buffer is a representative of several curves obtained during the use of assay buffer to perform serial dilutions. A one-site competition curve was attempted to fit the data by PRISM but could not be accurately accomplished, thus a Hill coefficient \( K_i \) could not be calculated. However, the IC\(_{50}\) was 4753 nmol/l. The calculated Hill coefficient for NEFAZODONE in buffer was 3.05.](image)

**TABLE 6**

<table>
<thead>
<tr>
<th>Nefazodone concentrations (( \mu \text{mol/l} )) in serial dilution tubes determined by HPLC( ^a )</th>
<th>5 mmol/l HCl</th>
<th>Assay Buffer( ^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>502.4</td>
<td>183.8 ± 35.2</td>
<td>72.0 ± 6.0</td>
</tr>
<tr>
<td>317.0</td>
<td>33.6 ± 2.6</td>
<td>17.5 ± 3.7</td>
</tr>
<tr>
<td>200.0</td>
<td>8.4 ± 0.9</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>126.2</td>
<td>3.9 ± 0.3</td>
<td>0.82 ± 0.04</td>
</tr>
<tr>
<td>79.6</td>
<td>1.7 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>50.2</td>
<td>0.82 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>31.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( a \) Serial dilutions performed in 5 mmol/l HCl were identical with those made in mobile phase. Only a single injection was tested for each concentration for each series of dilutions. \( n = 5 \) for each concentration.

\( b \) Assay buffer is that used for \textit{[3H]citalopram binding (table 1).}

Structural features significantly different from the other receptors (Arnold J. Mandell, personal communication). This portion of the \( m_1 \) receptor does not appear to be important in binding of “muscarinic” ligands (Brann \textit{et al.}, 1993).

Nefazodone is an effective antidepressant that is marketed as a serotonergic modulating agent that possesses a favorable side effect profile compared with the structurally related antidepressant, trazodone, and compared with the TCAs (Taylor \textit{et al.}, 1995). Unlike the SERT selective antagonists, nefazodone neither produces sexual dysfunction nor alters sleep architecture. Pharmacokinetic analyses find that, at steady-state concentrations, the molar hydroxynefazodone AUC is approximately 35% that of nefazodone. The molar AUC of mCPP is approximately 13% of that of nefazodone and that of the triazoledione tautomter is 160% that of nefazodone (Kaul \textit{et al.}, 1995; Mayol \textit{et al.}, 1994). Although nefazodone is not particularly potent \textit{in vitro} at antagonizing the SERT and NET, because of the high circulating plasma concentrations of drug, it can produce some transporter inhibition \textit{in vivo} (Hemrick-Leucke \textit{et al.}, 1994; Owens \textit{et al.}, 1995).

Based on metabolic patterns and affinity, the three major metabolites of nefazodone are unlikely to contribute to any transporter inhibition \textit{in vivo}. Nefazodone and hydroxynefazodone have similar affinity for all the other receptors tested and include potent affinity for the 5-HT\(_{2A}\) site which is thought to represent an important, although not well understood, aspect of its clinical effectiveness (Taylor \textit{et al.}, 1995). These two drugs are also quite potent at the \( \alpha \)-1 receptor, which is not consistent with the lack of orthostatic side effects and is one instance in which \textit{in vitro} binding affinity may not accurately predict potential side effects. The relatively potent affinity of nefazodone, hydroxynefazodone and triazoledione at the \( H_1 \) receptor are consistent with the sedative properties of nefazodone. Nefazodone and hydroxynefazodone possess moderate affinity for the \( \alpha \)-2 receptor. One could speculate that this may allow nefazodone to act, in part, in a manner similar to the new antidepressant mirtazapine on \( \alpha \)-2 auto- and heteroreceptors.

The TCAs exhibited a binding profile very similar to that reported previously. Thus, amitriptyline, imipramine, nortriptyline and desipramine showed high affinity for the SERT, particularly the human version, and for the NET in which the secondary amines were more potent. In agreement with these findings, amitriptyline and desipramine showed high affinity for the rat 5-HT\(_{1A}\) receptor, whereas nefazodone did not. However, nefazodone both antagonizes the rat 5-HT\(_{1A}\) receptor and has a binding affinity for the human 5-HT\(_{1A}\) receptor.
with previous data, the TCAs had high affinity for the H₁, 
alpha-1 and muscarinic receptors, which correlates well with 
their known side effect pattern of sedation, orthostatic hypo-
tension, dry mouth, constipation and tachycardia. One could 
also speculate that amitriptyline (table 3) and nortriptyline,
but not imipramine or desipramine (Cusack et al., 1994), may 
also act therapeutically via 5-HT₂A antagonism.

Venlafaxine, paroxetine, sertraline, citalopram, fluoxetine,
fluvoxamine and their various metabolites are all potent 
antagonists of the SERT. Although marketed as a “dual 
uptake inhibitor” (Effexor, package insert; Muth et al., 1986),
venlafaxine and O-desmethylvenlafaxine are not potent NET 
antagonists in vitro, although they do show activity in vivo.
This may be explained by the fact that free drug concentra-
tions in vivo may be relatively high because venlafaxine 
shows considerably less plasma protein binding (~20–25% 
bound) than any of the other compounds and, therefore,
likely antagonizes the NET as well as the SERT in vivo. Of 
the other non-TCAs tested, only paroxetine showed moder-
ately high affinity for the NET, although it was still 2 to 3 
orders of magnitude more potent at the SERT. However, as 
noted earlier, we have preliminary evidence that paroxetine 
administration in the high therapeutic range may affect the 
NET in vivo.

In general, the SERT selective antagonists were devoid of 
any meaningful potency at the various other receptors we 
examined with two exceptions. Paroxetine has moderately 
high potency for both muscarinic receptors (Kᵢ = 41 nmol/l) 
and the NET as noted above. This former finding is undoubt-
edly responsible for the dry mouth and occasional blurry 
vision observed with paroxetine use but it may reduce the 
frequency of diarrhea and loose stools that accompany the 
use of other SERT antagonists. The second exception is the 
moderately high potency of sertraline for alpha-1 receptors 
(Kᵢ = 36 nmol/l). Although one might predict significant 
orthostasis as a result, this has not been observed clinically,
perhaps because concentrations necessary for adequate 
SERT antagonism are considerably smaller than those 
needed for alpha-1 blockade.

The curves shown in figure 5 represent the curves we 
initially observed for several drugs in many different assays. 
Steep slopes are generally a sign of positive cooperativity 
which is observed with enzyme kinetics, but not typically in 
radioligand assays. We considered whether this was a solu-

tility issue; however, an incomplete dissolution of drug would 
indeed shift the curve to the right but would not change the 
shape of the curve as observed here. Because these drugs are 
all weak bases, even at the physiological pH of the buffers,
most of the drug would be in an ionized form and likely more 
soluble in the aqueous buffer than as the free base. Thus, the 
high degree of ionization produced by the 5 mmol/l HCl 
dilutions was probably not important for drug dissolution. All 
the compounds, with the exception of desmethylsertraline 
and mazindol, were easily solubilized in the 50% ethanol:50% 
5 mmol/l HCl solution at 1 mg/ml. Even when using assay 
buffer alone to perform serial dilutions, the dilution from 1 
mg/ml to the most concentrated dilution for the assay was 
200 to 250 μg drug/ml assay buffer and resulted in appar-
ently complete solubility. We further confirmed the fact that 
the drug was fully dissolved and not a particulate suspension 
by measuring drug levels directly from the serial dilution 
tubes where we observed a significant loss of drug (table 6 
and fig. 6). We further observed that addition to the assay 
buffer serial dilutions of 25 μl of 1 mol/l HCl directly before 
HPLC analysis resulted in a significant, but not complete,
recovery of drug that was observed to be “missing” in the 
dilutions of assay buffer alone (data not shown).

The apparent loss of potency and the steep competition 
curves could plausibly be explained by the presence of a 
saturable nonspecific binding site not affected by the si-
lanization process on the walls of the glass tubes. Although 
this is only speculative, this would mean that at lower drug 
concentrations all the drug is bound to this unidentified site,
which results in no competition for radioligand. At the point
where this site becomes saturated (i.e., significantly higher concentrations of serial drug dilution), there is now a large amount of drug available and considerable competition occurs. Because the data are plotted based on assumed concentrations, a very sharp drop in binding (i.e., steep curve) occurs. Whatever the mechanism, we believe that this may represent one explanation why in almost all instances in the future, unless every compound to be tested is examined comparing assay buffer versus dilute acid serial dilution, that serial dilutions for all drugs that are weak bases should be performed in dilute acid rather than assay buffer.

Acknowledgments

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References


Send reprint requests to: Michael J. Owens, Ph.D., Laboratory of Neuropsychopharmacology, Department of Psychiatry & Behavioral Sciences, 1639 Pierce Drive, Suite 4000, Emory University School of Medicine, Atlanta, GA 30322.