Tyr-95 and Ile-172 in Transmembrane Segments 1 and 3 of Human Serotonin Transporters Interact to Establish High Affinity Recognition of Antidepressants*

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L. Keith Henry**1, Julie R. Field15, Erika M. Adkins1, M. Laura Parnas1, Roxanne A. Vaughan1, Mu-Fa Zou**, Amy H. Newman**, and Randy D. Blakely15*

From the 1Department of Pharmacology and 2Center for Molecular Neuroscience, Vanderbilt University Medical Center, Nashville, Tennessee 37232, the 3Department of Pathology, Utah Medical Center, Salt Lake City, Utah 84112, the 4Department of Biochemistry and Molecular Biology, University of North Dakota, Grand Forks, North Dakota 58203, and **NIDA-Intramural Research Program, National Institutes of Health, Baltimore, Maryland 21224

In previous studies examining the structural determinants of antidepressant and substrate recognition by serotonin transporters (SERTs), we identified Tyr-95 in transmembrane segment 1 (TM1) of human SERT as a major determinant of binding for several antagonists, including racemic citalopram (RS-CIT). Here we described a separate site in hSERT TM3 (Ile-172) that impacts (RS)-CIT recognition when switched to the corresponding Drosophila SERT residue (I172M). The hSERT I172M mutant displays a marked loss of inhibitor potency for multiple inhibitors such as (RS)-CIT, clomipramine, RTI-55, fluoxetine, cocaine, nisoxetine, mazindol, and nomifensine, whereas recognition of substrates, including serotonin and 3,4-methylenedioxyamphetamine, is unaffected. Selectivity for antagonist interactions is evident with this substitution because the potencies of the antidepressants tianeptine and paroxetine are unchanged. Reduced cocaine analog recognition was verified in photoaffinity labeling studies using [125I]MZF 2-24. In contrast to the I172M substitution, other substitutions at this position significantly affected substrate recognition and/or transport activity. Additionally, the mouse mutation (mSERT I172M) exhibits similar selective changes in inhibitor potency. Unlike hSERT or mSERT, analogous substitutions in mouse dopamine transporter (V152M) or human norepinephrine transporter (V148M) result in transporters that bind substrate but are deficient in the subsequent translocation of the substrate. A double mutant hSERT Y95F/I172M had a synergistic impact on (RS)-CIT recognition (~10,000-fold decrease in (RS)-CIT potency) in the context of normal serotonin recognition. The less active enantiomer (R)-CIT responded to the I172M substitution like (S)-CIT but was relatively insensitive to the Y95F substitution and did not display a synergistic loss at Y95F/I172M. An hSERT mutant with single cysteine substitutions in TM1 and TM3 resulted in formation of a high affinity cadmium metal coordination site, suggesting proximity of these domains in the tertiary structure of SERT. These studies provided evidence for distinct binding sites coordinating SERT antagonists and revealed a close interaction between TM1 and TM3 differentially targeted by stereoisomers of CIT.

SERT, like several other members of the SLC6A gene family, acts to remove neurotransmitter from the synapse following neurotransmission (1) and is a major target for treatment of mood disorders, including major depression, anxiety, post-traumatic stress, and obsessive-compulsive disorders (2). Agents that target SERT include tricyclic antidepressants and serotonin-specific reuptake inhibitors (SSRIs) that block 5HT binding and uptake. Despite its clinical significance, very little is understood concerning the structural aspects of SERT and how they relate to its function and antagonist recognition. The current data predict that SERT proteins are composed of 12 transmembrane-spanning segments, intracellular NH2 and COOH termini and a large second extracellular loop bearing sites for N-linked glycosylation. Additionally, there is growing evidence that SERT forms a homomultimer in the plasma membrane, although most evidence suggests autonomous function of each monomer (3, 4).

The studies presented here utilize the strength of species variation at the SERT locus to direct our efforts in identification of residues that impact the structural and functional characteristics of the transporter. For example, in previous studies, we found that human and Drosophila SERT s possess distinct differences in recognition of mazindol and citalopram. To investigate these differences, human/Drosophila SERT chimeras were created and resulted in identification of TM1 and eventually a single residue, Tyr-95, as the residue responsible for these potency differences. Additional chimera studies looking at SERT substrates again identified TM1 and residue Tyr-95 as responsible for selective recognition of tryptamine analogs, particularly those with substitutions at the 4- and 7-position. A conserved Asp found in TM1 of all biogenic amine transporters also appears to play a role in substrate recognition and may also impact coupling (5). Recently, we performed a systematic analysis of TM1 of hSERT by using the substituted Cys accessibility method (SCAM) (6, 7). These studies implicated TM1 as forming part of an aqueous pore for 5HT permeation. Tyr-95, Asp-98, Gly-100, Asn-101, and Trp-103 appeared to form a critical stripe of amino acids that could be protected from MTS inactivation by 5HT co-incubation. Additional analyses revealed that aqueous accessibility of TM1 was lost at Val-97, a feature consistent with the cytoplasmic end of this domain contributing to an occluded pore. Together, these data strongly impli-
cate TM1 of SERT as an important contributor to substrate, ion, and inhibitor interactions.

In addition to TM1, TM3 has also been the subject of considerable structure/function studies. SCAM analysis revealed that TM3 plays a role in substrate and inhibitor binding. Specifically, residues Ile-172, Tyr-176, and Ile-179 were suggested to lie on one face of an α-helix with Ile-172 and Tyr-176 proximal to the binding sites for 5HT and cocaine (8). In addition to SCAM analysis, chimera and substitution mutagenesis in TM3 have implicated Met-180 as contributing to antidepressant interactions (9, 10).

Given the utility of analysis of species variants in SERT TM1, we examined TM3 for conspicuous changes that might support functional differences between Drosophila and human orthologs. We established a substitution of Met for Ile at TM3 172 within the region previously ascribed to contributing to substrate/antagonist recognition. We found that the hSERT I172M substitution has a dramatic impact on the potencies of many SSRIs, tricyclic antidepressants, and cocaine yet no impact on substrate recognition or translocation properties. Further characterization of I172M in the context of the Y95F substitution in TM1 supports the hypothesis that these two sites collaborate to establish high affinity CIT recognition. Moreover, differential recognition at Tyr-95 suggests that it alone is responsible for SERT stereoselective interaction with the R- and S-enantiomers of citalopram.

**Materials and Methods**

**Site-directed Mutagenesis and Construction of Mutant Plasmids—** Mutation of hSERT, mDAT, or hNET in pcDNA3.1 to generate the following mutants was performed using the Stratagene QuikChange kit as described previously (11): hSERT—Y95C, I172M, I172A, I172V, I172D, I172Q, I172F, I172R, I172K, I172Y, and I179C; mDAT V152M; and hNET V148M. Subsequent sequencing (Center for Molecular Neuroscience Neurogenomics and Sequencing Core Facility) confirmed the presence of only the intended mutations. The wild-type hSERT and I172M mutant cDNAs were also subcloned into the retroviral vector pBABE (12) to generate stable transformants in HEK-293 cells, achieved following selection in 2 μg/ml puromycin.

**SHT, DA, NE, and MPP⁺ Transport Measurements—** HEK and HEK-293 cells, maintained at 37 °C in a 5% CO₂, humidified incubator, were grown in complete medium (Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin). For initial evaluation of mutant transporter activity, cells were plated at a density of 100,000 cells per well in 24-well culture plates. Cells were transfected with SERT, NET, or DAT constructs with TransIT transfection reagent (Mirus Inc., 6 μl per μg DNA). Some transfected cells were grown in triplicate and repeated in three or more separate assays.

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**5HT Uptake Blockade by Cadmium in Cysteine Mutants—** Assays were performed as described above for transport measurements except that cells were incubated for 10 min with the indicated concentrations of CdCl₂ followed by addition of 5[3H]5-HT. Cell-incorporated label was determined by scintillation counting as detailed above. Counts were normalized to percentage of 5HT uptake with control and plotted versus cadmium concentration.

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**Western Blot and Surface Expression Analysis—** To determine total and surface expression of hSERT, mDAT, and hNET constructs were grown to confluency in 150 × 25-mm tissue culture-treated Petri plates. Medium was aspirated from cells, and cells were washed with ice-cold phosphate-buffered saline to detach from the plate and transferred to 50-ml conical tubes. Following low speed centrifugation, 700 × g, in a table top clinical centrifuge, supernatant was aspirated, and cells were resuspended in ice-cold TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) for cell disruption. The suspension was subjected to 20 strokes with a Dounce homogenizer to disrupt intact cells. Homogenate was transferred to cold 1.5-ml microcentrifuge tubes and centrifuged at 100,000 × g for 30 min (Sorvall Discovery M150 centrifuge). Supernatant was aspirated, and the pellet was resuspended in chilled binding buffer (50 mM Tris, pH 7.5, 100 mM NaCl). A portion of each sample was quantitated for protein content. For hSERT studies, [phenyl-6-3H]paroxetine (paroxetine; 19 Ci/mmol, PerkinElmer Life Sciences) (1.5 nM final) and cold competitor were combined in 12 × 75-mm borosilicate glass tubes. For hNET and mDAT studies, [125I]RTI-55 (2200 Ci/mmol, PerkinElmer Life Sciences) (0.5 nM final concentration) and cold competitor were combined in 12 × 75-mm borosilicate glass tubes. Binding buffer and membrane suspension were added, and tubes were gently mixed and allowed to incubate at room temperature for 1 h. Samples were processed as described previously (7). Data resulting from hSERT studies were analyzed by using one-site competition binding parameters on Prism 4 (Graphpad) to calculate Kᵢ values. Data from hNET and mDAT studies were expressed as femtomoles of RTI-55 bound and plotted using Prism 4.
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**Photoaffinity Labeling and Immunoprecipitation—**LLC-PK1 cells expressing rDAT and HEK-293 cells expressing hSERT or I172M hSERT were grown to 70% confluency in 6-well plates. Medium was removed, and 1 ml of [125I]MFZ 2-24 prepared in KRH buffer was added to a final concentration of 5 nM for 1 h at room temperature. For pharmacological studies, nonradioactive transporter ligands were included in the binding mixture at 1 μM final concentration. Cells were irradiated with shortwave ultraviolet light (254 nm, Foto-dyne UV Lamp model 3-6000) for 45 s at a distance of 15–20 mm to photoactivate the radioligand. Complete medium for HEK-293 cells or LLC-PK1 medium (α-minimum essential medium, 10% fetal bovine serum, 2 mM L-glutamine, and 100 μg/ml streptomycin) for LLC-PK1 cells was removed, and the cells were washed twice with 1 ml of ice-cold KRH buffer. The photolabeled cells were lysed with 0.5 ml/well RIPA buffer (50 mM NaF, 2 mM EDTA, 125 mM Na3PO4, 1.25% Triton X-100, and 1.25% sodium deoxycholate) for 45–60 min at 0 °C, followed by centrifugation at 20,000 × g for 15 min at 4 °C. The supernatant fraction was transferred to clean tubes for immunoprecipitation. Lysates were subjected to epitope-specific immunoprecipitation as described previously (15, 16) using antiserum 16 generated against amino acids 42–59 (LTNSTLINPPQTPVEAQE) of rDAT or antiserum 48 generated against amino acids 605–630 (KERIIKSITPETPTEIPCGDIRNAV) of rSERT. Immunoprecipitated samples were electrophoresed on 8% SDS-polyacrylamide gels followed by autoradiography using Hyperfilm MP film for 1–4 days at 80 °C.

**RESULTS**

**Multiple Sequence Alignment of TM3 from Various SERT Orthologs and Construction of hSERT I172M Mutant—**Based on our previous work indicating that (RS)-CIT and mazindol potency differences between *Drosophila* and human SERT were localized to TM1 and TM3 (11, 17, 18) as well as the previous suggestion that residues in TM3 are involved in substrate and inhibitor binding (8, 19), we sought evidence for divergent residues in TM3 that contribute to species-specific ligand recognition. The amino acid sequences of TM3 of SERT from various organisms were compared using the Megalign module within the DNAstar program package (Lasergene, Inc.) (Fig. 1). An alignment of TM3 of SERT from various organisms were compared using the Megalign module within the DNAstar program (Lasergene, Inc.) (Fig. 1). An alignment of TM3 of SERT from various organisms illustrates the high degree of amino acid identity present in TM3. Although the majority of SERTs we sampled have an Ile at the position homologous to Ile-172 in hSERT, a subset of insect species consisting of *Drosophila melanogaster* (fruit fly, Diptera), *Anopheles gambiae* (mosquito, Diptera), and *Manduca sexta* (tobacco hornworm, Lepidoptera) contain a Met at this position. Based on previous suggestions that Ile-172 in hSERT participates in both substrate and antagonist interactions (8), we proceeded to investigate the impact on hSERT function.

**FIGURE 1.** Protein sequence alignment of transmembrane domain 3 of monoamine transporters from various species. Alignment was produced using the Megalign module within the DNAstar program package (Lasergene, Inc.). The gray shaded amino acids represent the residue homologous to Ile-172 in hSERT. Residues shaded black represent species where the homologous residue is a methionine.

**FIGURE 2.** Analysis of hSERT I172M mutant function. A, relative activity of hSERT I172M was compared with wild type hSERT by determining the amount of radiolabeled 5HT transported into cells during a 10-min incubation. The data were normalized to the activity of wild type. B, saturation uptake analysis of SERT wild type and I172M mutant. HeLa cells transiently expressing wild type or I172M substituted SERT were monitored for uptake of radiolabeled 5HT. The resulting counts were converted to femtomoles/cell/min and plotted versus 5HT concentration that ranged from 0 to 5 μM. The data were fit to a Michaelis-Menten nonlinear regression curve using Prism 4.0 for Mac (Graphpad). C, analysis of expression levels of the I172M substitution. HeLa cells transiently expressing wild type or I172M were evaluated for SERT expression by Western blot analysis as described under "Materials and Methods." Nontransfected cells served as a negative control.
of Ile substitution with Met at this position in hSERT. The function of the hSERT I172M mutant construct was initially compared with hSERT using radiolabeled 5HT uptake and Western blot techniques with transiently transfected HeLa cells. Single time point uptake analyses revealed that both hSERT and mutant exhibited equivalent levels of $[^3H]5$-HT uptake (10 min, 20 nM) (Fig. 2A). Kinetic measurements of $[^3H]5$-HT uptake confirm indistinguishable rates of uptake between the hSERT WT and I172M (Fig. 2B). Consistent with these data, Western blot analyses of whole cell lysates from transiently transfected HeLa cells reveal the presence of relatively similar amounts of mature and immature SERT protein from hSERT or I172M (Fig. 2C). Saturation binding analysis of $[^3H]paroxetine$ revealed comparable $K_d$ values between mutant and wild type hSERT (0.4 versus 0.7 nM), data not shown.

FIGURE 3. Competition uptake analysis of SERT inhibitors and substrates. Cells expressing (top) hSERT (○) or hSERT I172M (■) or (bottom) dSERT (○) or dSERT M167I (■) were incubated with a constant concentration of $[^3H]5$-HT and increasing amounts of nonradioiodinated competitor as described under “Materials and Methods.” After 15 min, nontransported label was washed away; cells were lysed in scintillation fluid and assayed for radioactivity. Resulting counts/min were normalized to percent uptake of control well that lacked competitor. The normalized data were plotted versus log of the molar concentration of competitor. The data were fit to a nonlinear one-site competition curve.

A

B

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A

B
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**TABLE 1**

Impact of hSERT I172M mutant on SHT uptake inhibition constants for SERT substrates and inhibitors

| Substrates | Compounds | hSERT $K_i$ (m) | hSERT I172M $K_i$ (m) | Fold Δ |
|------------|-----------|----------------|----------------------|-------|
| 5HT        | 1.8 × 10^{-7} ± 0.30 | 1.8 × 10^{-7} ± 0.20 | 1.0 |
| tryptamine | 2.0 × 10^{-7} ± 0.09 | 2.0 × 10^{-7} ± 0.05 | 1.0 |
| 4HT        | 6.8 × 10^{-6} ± 0.80 | 5.8 × 10^{-6} ± 0.50 | 0.9 |
| α-MT       | 1.2 × 10^{-6} ± 0.50 | 1.4 × 10^{-6} ± 0.70 | 1.1 |
| 5HTM       | 2.1 × 10^{-6} ± 0.20 | 1.5 × 10^{-6} ± 0.20 | 0.7 |
| MDMAM       | 5.2 × 10^{-4} ± 1.50 | 6.5 × 10^{-4} ± 1.00 | 1.3 |
| 5MTM       | 4.2 × 10^{-2} ± 1.80 | 3.3 × 10^{-2} ± 1.40 | 0.8 |
| RU-24969   | 4.3 × 10^{-7} ± 0.04 | 9.8 × 10^{-7} ± 0.10 | 2.3 |
| MPP        | 7.0 × 10^{-6} ± 0.10 | 2.0 × 10^{-6} ± 0.01 | 0.3 |
| 7HT        | 2.8 × 10^{-6} ± 0.20 | 7.4 × 10^{-7} ± 1.10 | 0.3 |

Inhibitors

| Inhibitors | hSERT $K_i$ (m) | hSERT I172M $K_i$ (m) | Fold Δ |
|------------|----------------|----------------------|-------|
| tianepine  | 3.2 × 10^{-6} ± 0.80 | 2.8 × 10^{-6} ± 0.20 | 0.9 |
| amitriptyline | 7.6 × 10^{-8} ± 2.00 | 4.8 × 10^{-8} ± 1.50 | 1.6 |
| paroxetine | 5.5 × 10^{-10} ± 0.90 | 1.1 × 10^{-9} ± 0.03 | 2.1 |
| nomifensine | 3.3 × 10^{-6} ± 1.40 | 1.1 × 10^{-5} ± 0.50 | 3.3 |
| mazindol   | 3.2 × 10^{-7} ± 1.20 | 2.8 × 10^{-6} ± 0.20 | 8.8 |
| (RS)-fluoxetine | 6.7 × 10^{-9} ± 1.20 | 2.0 × 10^{-7} ± 0.20 | 29.9 |
| sertraline | 4.9 × 10^{-9} ± 0.50 | 1.6 × 10^{-7} ± 0.07 | 32.7 |
| cocaine    | 7.4 × 10^{-7} ± 2.60 | 5.1 × 10^{-5} ± 0.70 | 68.6 |
| (R)-citalopram | 1.8 × 10^{-7} ± 0.90 | 2.2 × 10^{-5} ± 0.40 | 120 |
| clomipramine | 2.3 × 10^{-6} ± 0.40 | 3.6 × 10^{-7} ± 1.70 | 157 |
| RTI-55     | 6.5 × 10^{-10} ± 2.10 | 1.2 × 10^{-9} ± 0.05 | 178 |
| (S)-citalopram | 4.5 × 10^{-8} ± 0.90 | 1.0 × 10^{-6} ± 0.50 | 752 |

*The following abbreviations are used: MDMA, 3,4-methylenedioxymethamphetamine; 5MT, 5-methoxyindolyltryptamine.

**TABLE 2**

Impact of mSERT I172M mutant on SHT uptake inhibition constants for SERT substrates and inhibitors

| Compound | Compounds | mSERT $K_i$ (m) | mSERT I172M $K_i$ (m) | Fold Δ |
|----------|-----------|----------------|----------------------|-------|
| 5HT      | 9.30 × 10^{-7} ± 0.8 | 1.20 × 10^{-6} ± 0.6 | 1 |
| 5MT      | 3.25 × 10^{-5} ± 3.3 | 4.73 × 10^{-5} ± 1.6 | 1 |
| tyramine | 1.20 × 10^{-4} ± 0.3 | 7.80 × 10^{-5} ± 0.5 | 1 |
| (RS)-fluoxetine | 4.20 × 10^{-4} ± 3.0 | 1.80 × 10^{-6} ± 0.6 | 43 |
| (S)-citalopram | 5.00 × 10^{-9} ± 0.2 | 6.85 × 10^{-6} ± 1.5 | 1370 |
| clomipramine | 6.15 × 10^{-10} ± 5.3 | 9.45 × 10^{-7} ± 3.6 | 1537 |

**TABLE 3**

Impact of hSERT I172M on inhibition constants for competition of $[^3H]$paroxetine binding

| Compound | hSERT $K_i$ (ms) | hSERT I172M $K_i$ (ms) | Fold Δ | Fold Δ from uptake assay |
|----------|----------------|----------------------|-------|-------------------------|
| cocaine  | 1300 ± 360 | 51,300 ± 12,000 | 39 | 69 |
| (R)-citalopram | 810 ± 74 | 47,000 ± 5200 | 58 | 120 |
| amitriptyline | 310 ± 140 | 24,800 ± 2000 | 80 | 1.6 |
| clomipramine | 176 ± 11 | 470 ± 310 | 157 | 52 |
| (S)-citalopram | 14 ± 0.3 | 14,500 ± 5000 | 1021 | 752 |
| (R)-citalopram | 23 ± 9.6 | 24,000 ± 12,000 | 1043 | 1240 |

Effect of I172M on Potency of Substrates and Inhibitory Compounds—To evaluate the pharmacological consequences of the I172M mutation, the $I_{50}$ values for $[^3H]$5-H T uptake inhibition for various neurotransmitter substrates and inhibitors were determined. All the substrates tested displayed little or no change in their ability to compete for uptake of 5HT in hSERT versus I172M mutant-expressing cells (Fig. 3 and Table 1). Similarly, the $K_m$ values (14 μM WT versus 12 μM I172M) for transport of the neurotoxic substrate MPP$^+$ did not change with the Ile to Met substitution. Most surprisingly, more than half of the nontransported inhibitors of SERT showed decreases in potency in the I172M background ranging from 9-fold for mazindol to >10,000-fold loss for (R)-CIT. Additionally, cocaine and its structural analog RTI-55 displayed 69- and 178-fold losses in potency, respectively. Most interestingly, a few compounds like the SSRI paroxetine and the tricyclic antidepressants amitriptyline exhibited less than a 2.5-fold change in potency.

Reciprocal Mutation of the Ile-172 Homologous Residue Met-167 in dSERT to Ile Results in a Gain of Function Phenotype in Regard to Inhibitor Potency—To validate the unique ability of the I172M substitution to impact inhibitor potencies to such a large degree, the same mutagenesis methods described earlier were employed to convert the dSERT residue Met at position 167 (homologous to Ile-172 in hSERT) to Ile. The mutant was then evaluated by $[^3H]$5-HT competition uptake assays with either unlabeled 5HT or antagonists. As seen in the hSERT I172M mutation, 5HT (2.9 → 3.1 μM) and paroxetine (26 → 9.7 nM) potencies showed little to no change in the dSERT M167I background (Fig. 3, bottom). However, cocaine (22 → 0.37 μM), fluoxetine (840 → 25 nM), and (R)-citalopram (400 → 9.3 nM) showed marked increases in potency in the mutant dSERT compared with native dSERT.

Characterization of I172M Substitution in Mouse SERT—We wanted to extend the analysis to rodent SERTs to determine whether the impact of the substitution was compatible with future in vivo manipulations in a murine model. To evaluate this idea, we expressed mSERT I172M in HeLa cells and performed competition uptake studies. The compounds tested yielded results that were strikingly comparable with those seen with the hSERT mutant (Table 2). As with the hSERT mutant, none of the substrates tested were impacted by Met substitution. Potency losses for (R)-CIT and fluoxetine were comparable with those seen in hSERT.
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Most interestingly, clomipramine exhibited a 10-fold greater loss in potency in mSERT compared with hSERT, suggesting somewhat distinct conformations or binding contacts for tricyclics at hSERT versus mSERT.

**Competitive Binding Analysis of the hSERT I172M Mutation Yields Similar Results as Competitive Uptake Assays**—Loss of antagonist recognition at the I172M substitution could arise from a modification of ligand-binding sites or be indirect via long range conformational perturbations linked to transport. Therefore, we wanted to confirm that loss of potency with the I172M substitution reflects a concomitant loss of binding affinity, and thus we employed \(^3\)H]paroxetine competitive binding assays (Table 3). \(^3\)H]Paroxetine was used to label SERT as the SSRI showed little to no change in apparent \(K_i\) values for either hSERT or I172M SERT (Fig. 3). Our results revealed binding trends similar to those observed with the uptake inhibition studies. For instance, (S)-CIT, which showed a 750-fold loss in potency for the I172M mutant, exhibited a comparable 1000-fold reduction in potency for displacement of \(^3\)H]paroxetine binding for I172M versus hSERT. Similarly, (R)-CIT (120- versus 58-fold), cocaine (69- versus 39-fold), and clomipramine (157- versus 276-fold) showed comparable fold loss values for inhibition of 5HT uptake and paroxetine binding comparing hSERT and the I172M mutant. One exception was amitriptyline, whose potency for 5HT inhibition was unaffected by the Met substitution but showed a modest 80-fold loss in potency for \(^3\)H]paroxetine binding.

**SERT Photoaffinity Labeling by MFZ 2-24 Is Lost in the I172M Mutant**—In order to validate further an influence of the I172M mutant on antagonist interactions, we employed the photoaffinity label MFZ 2-24 (cocaine analog) (20). Similar photoaffinity labels have been used with success to probe domains involved in recognition of DA uptake antagonists in DAT (21). In uptake inhibition studies, MFZ 2-24 exhibited a reduced potency (120-fold) at hSERT I172M relative to hSERT (data not shown). HEK-293 cells expressing hSERT were subjected to photoaffinity labeling with \(^{125}\)I]MFZ 2-24 in parallel with LLC cells expressing rDAT as a positive control. Prominently labeled bands of 70–80 kDa were visible in detergent lysates from both cell lines, and immunoprecipitation of lysates with corresponding anti-DAT or anti-SERT antibodies specifically extracted radiolabeled proteins with established DAT and SERT molecular masses (Fig. 4A). DAT and SERT were labeled relatively equally by \(^{125}\)I]MFZ 2-24, consistent with a similarity between the transporters in the mechanism of MFZ 2-24 binding and labeling. The pharmacological specificity of SERT photolabeling with \(^{125}\)I]MFZ 2-24 was verified by including 5HT reuptake inhibitors with the radioligand prior to photoincorporation. Covalent labeling of SERT was strongly reduced by the SERT blockers (–)-cocaine, citalopram, imipramine, and fluoxetine, but not by (+)-cocaine or the DAT and NET blockers GBR 12909 and nisoxetine (Fig. 4B). These results demonstrate that \(^{125}\)I]MFZ 2-24 photoaffinity labels SERT and show that the irreversible incorporation sites on DAT and SERT for this compound are likely to be similar. The effect of SERT mutation I172M on photoaffinity labeling is shown in Fig. 4C. Equal amounts of hSERT or mutant cells were incubated with \(^{125}\)I]MFZ 2-24 followed by ligand activation and SERT immunoprecipitation. Autoradiographs of detergent lysates showed a prominent band corresponding to SERT in the WT cells but only background staining in the I172M cells. Similarly, after immunoprecipitation, the WT SERT protein labeled with \(^{125}\)I]MFZ 2-24 is visible, but no labeling of SERT I172M was detectable. Control Western blots demonstrated that I172M does not disrupt immunoprecipitation, supporting a specific loss of labeling (data not shown). These results documented the utility of \(^{125}\)I]MFZ 2-24 for SERT labeling and showed that the loss of reversible cocaine binding found for the I172M mutant is paralleled by a loss of irreversible cocaine analog binding.

**Methionine Appears Unique in Its Influence at Ile-172 in hSERT**—We evaluated the requirement of Met for antagonist and substrate discrimination by substituting several other amino acids in place of Ile at position 172 and assessing their activity and pharmacology (Fig. 5, A and B). The amino acid substitutions, chosen based upon reactivity, charge, and size characteristics, were as follows: Ala, Cys, Asp, Phe, Met, Gln, Thr, Arg, Lys, and Val. Statistically significant increases (\(p < 0.05\)) in the apparent \(K_i\) values for 5HT were observed for the Asp, Gln, and Phe substitutions, whereas the citalopram \(K_i\) increases were seen with Met, Asp, Gln, and Phe. Both 5HT and (RS)-CIT potencies seemed to be strongly affected by both charge and bulk. However, Ile, which is rather bulky (similar in volume to Met and Phe), is not detrimental to (RS)-CIT potency. Substitutions with the charged amino acids Arg and Lys yielded transport-inactive transporters and were not further analyzed.

**Analysis of Homologous Mutations in mDAT and hNET**—To determine whether a similar impact on ligand recognition could be achieved in other monoamine transporters, the homologous sites in mDAT (Val-
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[FIGURE 5. Impact of amino acid substitution at Ile-172 on citalopram potency. HeLa cells expressing the various substitutions at residue 172 were evaluated by competitive uptake analysis as described under “Materials and Methods” to determine \( K_i \) values for inhibiting 5-HT uptake. The resulting \( K_i \) values were plotted against residue size expressed in van der Waals volume (A) and calculated hydrophilicity as determined by partitioning coefficient between water and octanol (B) (31). Arrows indicate increasing volume or hydrophilicity.

152) and hNET (Val-148) were mutated to create mDAT V152M and hNET V148M. Both mutants were expressed and successfully trafficked to the plasma membrane similar to that of mDAT and hNET, respectively, as verified by detection of biotinylated surface protein (Fig. 6, A and B). However, radiolabeled uptake assays using tritiated DA and NE revealed that the Met substitutions in hNET and mDAT resulted in complete loss of substrate transport (Fig. 6, C and D). Testing for uptake of \([^3H]5\text{-HT}\) revealed that conversion of this residue to the dSERT identity did not result in promiscuous transport of 5-HT (data not shown). Although substrate translocation was abolished in these mutants, it was possible that substrate binding remained intact even if at a lower affinity. To evaluate this aspect of transporter-substrate interaction, we performed a single concentration \([^{125}\text{I}]\text{RTI-55}\) competition binding assay by using membranes isolated from cells expressing hNET, mDAT, or mutant transporters to determine whether substrate could compete for a DAT/NET antagonist. The results (Fig. 6E) show that, as with hNET and mDAT, RTI-55 binding to both the hNET V148M and mDAT V152M mutants could be specifically inhibited by excess DA. We suspected that RTI-55 binding could be impacted by these mutations. This assumption was substantiated by comparing the relative amount of RTI-55 bound in samples minus 1 mM DA. RTI-55 binding appears to be reduced 50 and 66% for hNET V148M and mDAT V152M, respectively, compared with the native transporters. More importantly, even though RTI-55 affinity may be affected to some degree, the data clearly showed that substrate can specifically inhibit antagonist binding to the mutant transporters indicating the mutants are capable of binding substrate but lose the capacity to complete substrate translocation.

Analysis of a Single Y95F and a Double Y95F/I172M Mutant Reveals Synergistic Support for (S)-Citalopram Potency Identifying Differential Potency Determinants for (R)- Versus (S)-Citalopram—Our previously published studies suggest an interaction between the inhibitors citalopram and mazindol and residue Tyr-95 in hSERT (11). As Met substitution at Ile-172 significantly impacted (RS)-CIT potency, we investigated the possible interaction between Tyr-95 and Ile-172 to coordinate (RS)-CIT binding. The double mutant was slightly reduced in 5HT uptake efficiency similar to that of the single Y95F substitution as determined by \([^{3H}]5\text{-HT}\) uptake (data not shown). This lack of further impact on 5HT transport in the double mutant background suggests little or no impact on gross structure beyond that of the Y95F change. Competitive uptake assays with racemic and R- and S-isomers of citalopram were performed with the double and single mutants to investigate possible stereoselectivity and/or cooperativity involving these residues in citalopram binding. The potency of 5HT to inhibit its own uptake was unchanged in single and double mutants compared with wild type (Fig. 7). Remarkably, (RS)-citalopram potency was decreased by ~10,000-fold in the double mutant. This synergistic effect of both mutations on CIT potency suggests that these two determinants may collaborate to support binding of the clinically active enantiomer (S)-CIT. In contrast, (R)-CIT, whose potency was significantly reduced by the I172M substitution (117-fold), showed only a small 5-fold change in potency when tested in the Y95F background. Furthermore, (R)-CIT potency in the double mutant was similar to that seen with the I172M single mutant (117- versus 79-fold, respectively), suggesting (R)-CIT is not greatly influenced by the Y95F substitution.

In contrast to the small effect of the Y95F mutant on (R)-CIT potency, the Y95F mutation displayed a considerable reduction in the potency of both (RS)-CIT and (S)-CIT (37- and 19-fold, respectively). However, the loss of potency observed with the single I172M mutant (867- and 344-fold) suggests Ile-172 as the major contributor to high affinity interaction between hSERT and (S)-CIT.

Analysis of the double mutant resulted in an even greater loss of potency for (S)-CIT and (RS)-CIT (6,000- and 9,200-fold) indicating the concerted contribution of both mutations to (S)-CIT binding. (S)-CIT and (RS)-CIT exhibited similar potency profiles to one another for hSERT and the mutant transporters indicating the (R)-CIT/(S)-CIT potency disparity is masked by the comparatively high affinity interactions of (S)-CIT. As reported previously (11), the tricyclic antidepressant-related stimulant mazindol exhibited a 2-fold increase in potency in hSERT Y95F mutant compared with WT. When tested with the hSERT I172M mutant, mazindol exhibited a 7-fold loss in potency. hSERT I172M mutant, mazindol exhibited a 7-fold loss in potency versus WT. However, this potency loss was attenuated when Y95F was introduced into the I172M background and resulted in a 2-fold gain in potency compared with the I172M substitution alone. This finding indicates the positive impact of Phe at position 95 was not abolished in the double mutant and further validates that the mutations are not grossly affecting transporter structure/function.

Engineered Cysteines in TM1 and TM3 Form Coordination Site for Cadmium—As the mutagenesis and pharmacological data in this study suggested TM1 and TM3 are involved in antagonist binding, we investigated whether TM1 and TM3 were proximal to one another in the tertiary structure of SERT. To test this hypothesis, we replaced several residues within TM1 and TM3 with cysteine and probed these mutants with cadmium to determine whether any of the mutated residues were close enough to coordinate cadmium binding. The residues were chosen based upon interpretation of our previous SCAM analysis of TM1
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and pharmacological data from TM1 and TM3 mutants. Two double mutants were constructed in the cadmium-insensitive hSERT C109A background, hSERT Y95C/I172C and hSERT N101C/I179C. The double and respective single mutants were assayed for dose-dependent inhibition of 5HT uptake by cadmium (Fig. 8, A and B). The immortal form represents non-glycosylated transporter and makes up a smaller proportion of the transporter on the surface. Introduction of I172C results in only slight sensitivity to cadmium (40% reduction in uptake at highest cadmium concentration). Greater sensitivity was seen in the Y95C mutation; however, no increased sensitivity was seen in the Y95C/I172C double mutant compared with Y95C alone. This suggests that I172C and Y95C are not close enough to cooperatively coordinate cadmium. However, results with the second set of cysteine mutants were significantly different. The single N101C and I179C mutants showed increased sensitivity to cadmium similar to that seen with the Y95C single mutant. Remarkably, sensitivity to cadmium in the hSERT N101C/I179C double mutant was increased ~4-fold based on the concentration of cadmium necessary for 50% inhibition of 5HT transport. These data suggest that Asn-101 in TM1 and Ile-179 in TM3 are sufficiently proximal to form a high affinity coordination site for cadmium. Furthermore, these data are consistent with the idea that TM1 and TM3 are close enough in the folded structure of the transporter to form part of an antagonist-binding site.

**DISCUSSION**

Evidence of phenotypic differences between homologous proteins from different species provides a powerful platform for elucidating res-
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FIGURE 7. Effect of Y95F and I172M mutations in hSERT on potency of 5HT, citalopram, and citalopram enantiomers and mazindol. A, a chemical structure illustration of (R)- and (S)-citalopram. B, HeLa cells expressing the hSERT Y95F, I172M, or Y95F/I172M mutants were assayed using a 5HT competition uptake assay as detailed under “Materials and Methods.” The resulting log \( K_i \) values of 5HT, citalopram, and its enantiomers are presented in bar graph form. The arrow indicates increasing potency. An unpaired, two-tailed \( t \) test was performed between the WT in each mutant using Prism 4.0 for Mac. An additional analysis comparing Y95F to the Y95F/I172M was also carried out and is denoted by braces. The statistical results are indicated as follows: *, \( p < 0.05; **, p < 0.001; ns, not significant.

We have identified a Drosophila/human variation in TM3 at residue Ile-172 in hSERT that upon conversion to the Drosophila Met has no discernible effect on 5HT translocation but results in a dramatic reduction of potency (up to 1000-fold) for the SSRI (RS)-CIT. However, this dramatic shift in potency was not unique to CIT, as rather large decreases in potency were observed with other SSRIs, tricyclic antidepressants, cocaine, and cocaine analogs. A few SSRIs and tricyclic antidepressants failed to exhibit a potency shift. This lack of potency change with a subset of antagonists likely reveals different binding requirements for distinct classes of inhibitors. For instance, the SSRI paroxetine, one of the compounds whose potency did not change in response to the I172M substitution or modification at Tyr-95 (11), has been suggested to alter cocaine binding to SERT via an allosteric interaction rather than direct competition (24). More importantly, further pharmacological testing of the mutant SERT with regard to substrates revealed that, in addition to 5HT, no observable change in potency was seen with any of the substrates we tested. These data suggest the possibility of a direct interaction between Ile-172 and affected inhibitors that is absent with substrates. However, substitutions other than Met negatively impacted 5HT potency, although not as profoundly as (RS)-CIT potency. Side chain bulk and charge seemed to have a proportional disruptive effect on (RS)-CIT potency with the exception of Ile, which itself is a relatively bulky group. The ability of Ile to accommodate inhibitor binding despite its bulk may be because of the branched nature of the Ile side chain similar to Val found at this position in NET and DAT.

We find these results compelling especially when considered along with previous data concerning the characteristics of the Ile-172 residue in SERT (8) which showed the following. 1) The activity of the mutant I172C was disrupted by incubation with MTSET, implying Ile-172 faces an aqueous environment, accessible to externally applied MTS reagents. 2) Inactivation of I172C by MTSET was blocked by co-incubation with cocaine or 5HT, suggesting Ile-172 is at or near the cocaine-binding site and the 5HT permeation pathway. 3) Remarkably, I172C modification by MTSET reversed over time, indicating Ile-172 might interact with both the extracellular and reducing intracellular environment, suggesting possible roles in a gating mechanism or positional movement within the transporter during translocation. In contrast, the Ile-179 located two helical turns from, and almost directly above, Ile-172 (Fig. 9) could be inactivated by MTSET, whereas the MTSET-treated mutant still bound β-CIT and 5HT, indicating Ile-179 is at most indirectly involved in the binding site for substrate and inhibitor. The authors concluded that Ile-172 was likely close to a binding site shared by both 5HT and cocaine. Another study by Mortensen et al. (9) in which Met-180 in TM3 of hSERT was substituted with the isoleucine found in bovine SERT resulted in no shift in potency for cocaine, sertraline, or RTI-55. However the mutant SERT did show moderate (2–4-fold) changes for imipramine, fluoxetine, paroxetine, and citalopram (2–3-fold), which in our study would represent a more minimal change compared with the impact of I172M. Unlike I172M, M180I did not alter selectivity for cocaine, sertraline, or RTI-55, which may indicate these compounds bind deeper in the transporter. Additionally, Met-180 was inaccessible to MTS reagents, suggesting it is buried in a hydrophobic environment. Based on a helical model of TM3, Met-180 is found almost 90° around the helix from Ile-172 and seems to play a smaller role in substrate and ligand recognition in SERT. In contrast, Ile-172, which is MTS-accessible, seems to face an aqueous environment even though the face of the helix on which Ile-172 resides is strongly hydrophobic. The hydrophobic nature of this region of TM3 may of course not always be exposed, such as during the transport cycle. The side opposite I172M on the helix is quite charged and might have been expected to be aqueous-accessible but may utilize polar residues to facilitate helix packing (26). Given the importance of I172M on antagonist recognition, we initiated studies to understand the influence of the I172M substitution on...
substrate recognition and selectivity. Initial studies focusing on structurally diverse tryptamine analogs have yet to provide evidence of specific interactions between Ile-172 and substrates. The structural analogs we have tested in this study consist of adducts or substractions to the following: 1) the benzene ring of the indole (5-hydroxy-7-methoxytryptamine); 2) the α-carbon of α-methylserotonin; 3) positions R3 and 5 with 5-methoxy-N-isopropyltryptamine; and 4) position 5 and a bulky cyclic group to the β-carbon in RU24969. Additional studies with structurally dissimilar analogs are underway. Most interestingly, the homologous residue when mutated has a profound impact on substrate binding sites for cadmium. HeLa cells expressing hSERT or selected hSERT cysteine mutants were subjected to increasing concentrations of CdCl2 and assayed for [3H]5-HT accumulation as described under “Materials and Methods.”

Radiolabeled competition and uptake assays suggest cooperativity between Tyr-95 and Ile-172 for high affinity citalopram binding to hSERT and specifically point to Tyr-95 as a major contributor for high affinity binding of (S)-CIT. In competition binding studies using [3H]paroxetine to analyze binding properties of the I172M mutant, the binding affinity of tested compounds was comparable with potency shifts observed in the uptake assays. One exception was the tricyclic antidepressant, amitriptyline, which showed an 80-fold change in binding affinity compared with only a 2-fold change in the 5HT competition uptake assay. Discrepancies between uptake and binding inhibition assays have been noted previously (7, 9) and may represent a different impact of the assays on SERT structure and/or accessibility. The uptake assays are performed on whole cells, whereas purified membranes are used in the binding competition assays. Whole cell uptake assays with labeled paroxetine were attempted but yielded unacceptable nonspecific binding.

We focused on possible intramolecular interaction between Ile-172 and other SERT residues. Previous species variation studies by our laboratory in TM1 of SERT revealed Tyr-95 interacts with (R)-CIT and mazindol, and as both compounds were affected by the I172M mutation, we investigated the impact of inhibitor potency in SERTs containing both mutations. Mazindol potency was reduced in the double mutant but not to the degree seen in the single I172M mutant, suggesting that the previously observed positive effect of Phe at position 95 was retained in the double mutant. This observation and the lack of change in 5HT potency is further evidence that these substitutions do not grossly affect transporter structure or function. Pharmacological testing of the single and double mutants revealed a striking observation. The residue Tyr-95 appears to be largely, if not solely, responsible for the potency differences observed between (R)- and (S)-CIT. Based on these data, we propose that the R-isomer exists in a structure that rotates an interacting group away from critical interaction with Tyr-95 resulting in loss of a binding contact, whereas the S-isomer is able to make an appro-
priate contact with Tyr-95 resulting in increased binding and potency. Possibly, Tyr-95 interacts with either the 3-(dimethylamino)propyl or 4-fluorophenyl moieties that make up the chiral arms of CIT. Alternatively, the R-isomer may interfere stereically with interaction of Tyr-95 with the ether oxygen on (RS)-CIT, an interaction previously proposed by Barker et al. (11). The Y95F mutant is the first substitution to impact stereospecific recognition of an inhibitor by SERT and could provide a critical tool in studying the fundamentals of citalopram-SERT interactions. More importantly, there are emerging data that allosteric modulation of (S)-CIT binding by the R-isomer may influence in vivo potency for uptake blockade by the racemic mixture (28, 29). In contrast, the I172M mutation impacts the potency of both (R)- and (S)-CIT equivalently, suggesting that Ile-172 interacts with a region of citalopram that is not affected by isomerization, i.e. the 1,3-dihydroiso-benzofuran-5-carbonitrile group. Met-180, also in TM3, has been reported to interact with the heterocyclic portion of this group (9). (R)- and (S)-CIT collapsed to a unitary low potency value in the Y95F/I172M double mutant. The remaining low affinity binding is suggestive of an additional binding contact(s) yet to be identified. However, it is also possible that the remaining affinity for the SERT mutant represents a nonelective interaction shared among DAT, NET, and SERT. It would be interesting to determine whether (R)-CIT retains allosteric modulation of (S)-CIT binding in the double mutant where each has equivalent binding.

In contrast to CIT isomers, competition uptake studies of the purified isomers of fluoxetine ((R)-fluoxetine and (S)-fluoxetine) with the I172M and Y95F mutants indicate the potency of both isomers was negatively impacted by the Y95F and I172M mutations. However, neither isomer demonstrated a selective impact by one or the other (data not shown). This is not surprising because unlike (RS)-CIT, fluoxetine isomers are not known to have any clinical differences from one another or the racemic fluoxetine (30).

Moreover, the loss of potency for cocaine and cocaine-like structures for the I172M mutant was further validated in studies using the photoaffinity, radioiodinated MFZ 2-24 compound. MFZ 2-24 failed to label the mutant and supported the involvement of Ile-172 in cocaine and cocaine analog binding. To our knowledge, these assays represent the first demonstration of specific photoaffinity labeling of SERT. The equivalent labeling intensity of DAT and SERT by MFZ 2-24 suggests a similar binding pocket and site of covalent attachment and may indicate that MFZ 2-24, which incorporates in TM1 or TM2 of DAT, could photolabel similar domains in SERT. In contrast, RTI-82, which labels TM4–6 of DAT (9), does not incorporate into SERT as well as it does DAT (data not shown), suggesting inherent structural/contact differences between DAT and SERT as revealed by this molecule.

In an attempt to gain more insight into the structure of monoamine transporters, some studies have used molecular modeling and substrate docking software in combination with current biochemical data to model transporter helix packing and ligand binding interactions. One such report by Ravna et al. (32) uses the Escherichia coli Na+/H+ antiporter NhaA electron density projection map as a basis for helix order and packing of SERT. Cocaine and (R)- and (S)-citalopram were docked into the resulting energy-minimized structure by using current biochemical data regarding putative interaction sites. By using several docking positions, the authors posit that the difference in (R)- and (S)-citalopram binding was because of the inability of (R)-citalopram to interact with the residue Ile-172. However our competitive uptake data strongly suggest that (R)- and (S)-citalopram both interact with Ile-172, but only (S)-citalopram can make the additional contact with Tyr-95 resulting in its increased potency. These data indicate SERT helix packing may be distinct from NhaA and could help in refining future in silico models of SERT-ligand interactions. However, the results also suggest caution in use of nonhomologous proteins to construct structural models.

The striking effect of the I172M mutant raises questions as to possible structure/function correlates among the monoamine transporters at this site. Here we showed that homologous mutations to I172M in hNET and mDAT resulted in transporters that were unable to translocate radiolabeled DA, NE, or 5HT, even though the mutant transporters were successfully trafficked to the cell surface. However, DA displacement of RTI-55 in both mutant transporters indicates the mutants retain substrate binding but lack the ability to complete the translocation step. This finding is further supported by studies with the fluorescent NET substrate ASP+ (33) (data not shown). In these studies, the NET mutant was able to bind ASP+ but unlike wild type could not translocate the fluorophore inside the cell. These observations lead to several possibilities. NET and DAT may be structurally/functionally distinct from their family member SERT at TM3, an idea that has been suggested by MTS studies with DAT (34), or this particular residue is more critical to substrate interaction in catecholamine transporters. Although both the NET and DAT mutants were found to be binding-competent for substrates, our study does not address whether the binding affinity is altered. However, as this substitution results in transporters unable to move substrate into the cell, it suggests a role of these residues in some type of translocation mechanism. Two studies by Lee et al. (27, 35) suggest that TM3 is crucial for function in DAT and identify residue Val-152 in hDAT (homologous to hSERT I172), which upon mutation to Ile (found in bDAT) greatly reduced recognition of 2β-carbomethoxy-3β-(4-fluorophenyl)tropane and uptake of the substrates DA and MPP+. Most interestingly, mutation of hDAT Val-145 to Ala was found to uniquely alter MPP+ transport, whereas DA uptake was spared, suggesting some substrate selectivity may be influenced by residues proximal to Val-152 (Ile-172). Unlike the substitution of Val-152 in DAT, our substitution of Ile-172 did not influence MPP+ transport kinetics. However, we did not investigate the SERT residue that correlates to hDAT Val-145 for its impact on substrate recognition. These observations warrant strong consideration for further studies of TM3 residues in NET, DAT, and SERT for their role in substrate selectivity and antagonist binding.

During the review process for this work, a high resolution crystal structure (Protein Data Bank code 2A65) for LeuTaa, a leucine bacterial transporter and ortholog of the monoamine transporters, was reported (36). The structure was solved with leucine in the purported binding pocket. In the LeuTaa structure, Val-104, which is orthologous to Ile-172 in hSERT, Val-148 in hNET, and Val-152 in mDAT, is directly involved in formation of a hydrophobic binding site that accommodates the hydrophobic methyl groups of the leucine side chain. Furthermore, hSERT Tyr-95 is orthologous to the LeuTaa residue Asn-21 that the authors report is directly involved in binding of leucine. Therefore, the LeuTaa crystal structure and our biochemical and pharmacological data concerning the proximity and function of hSERT Tyr-95 and Ile-172 are in complete agreement and support the veracity of each study. Most surprisingly, TM1 and TM3 in LeuTaa interact only across a small region as TM3 is tilted 55° in relation to the lipid bilayer. Remarkably, the residues we chose to test for formation of a high affinity cadmium site through cysteine introduction all fall within this small region of interaction; however, the success of only one set of cysteine mutants may be because of side chain accessibility. Furthermore, based on the finding that addition of only Y95C, N101C, or I179C results in increased

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3 R. Vaughan and L. Parnas, unpublished results.
cadmium sensitivity, we propose that residue Asp-98, which lies one helical turn below Asn-101 and one helical turn above Tyr-95, participates in the formation of the engineered cadmium-binding site. However, this proposal is difficult to test empirically as Cys substitutions at Asp-98 are not well tolerated by the transporter. However, the position of the hSERT D98 orthologous residue in LeuTα strongly supports our idea that Asp-98 contributes to the formation of the cadmium-binding site. These data are in agreement with a recent report using this methodology to provide evidence of proximity of TM1 and TM3 in the tertiary structure of a transporter with the same family as SERT, GAT1 (25).

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