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Comparative molecular field analysis using selectivity fields reveals residues in the third transmembrane helix of the serotonin transporter associated with substrate and antagonist recognition

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Abstract

The human serotonin transporter (hSERT) regulates the spatial and temporal actions of serotonin (5-HT) neurotransmission by removing 5-HT from the synapse. Previous studies have identified residues in the third transmembrane helix (TMH) that may be important for substrate translocation or antagonist recognition. We identified hSERT residues in TMH III that are divergent from *drosophila* SERT (dSERT) and employed species-scanning mutagenesis to generate reciprocal mutants. Transport inhibition assays suggest that the potency of substituted amphetamines was decreased for the hSERT mutants A169D, I172M, and S174M. Additionally, there was a loss of potency for several antidepressants and 3-phenyltropane analogs for the I172M mutant. These results suggest that residues in TMH III may contribute to antagonist recognition. We carried out Comparative Molecular Field Analyses (CoMFA) using selectivity fields to directly visualize the mutation-induced effects of antagonist potency for antidepressants, 3-phenyltropane analogs, and amphetamines. The hSERT I172M selectivity field analysis for the 3-phenyltropane analogs revealed that electrostatic interactions resulted in decreased potency. The amphetamine and antidepressant selectivity field analyses reveal the observed decreases in potencies for the hSERT I172M mutant are due to a change in tertiary structure of the hSERT protein and are not due to disruption of a direct binding site. Finally, the hSERT mutant A169D displayed altered kinetics for sodium binding, indicating that this residue may lie near the putative sodium-binding site. A SERT homology model developed from the *Aquifex aeolicus* leucine transporter structure provides a structural context for further interpreting the results of the TMH III mutations.

INTRODUCTION

The serotonin transporter (SERT) is a member of the Na⁺/Cl[−]-dependent neurotransmitter family of transporters and functions to terminate the neurochemical signal transmitted from the presynaptic neuron following an action potential (Barker and Blakely, 1995). The transport of a molecule of serotonin (5-hydroxytryptamine, 5-HT) into the presynaptic neuron is thought to be electrogenically coupled to the inward transport of one molecule each of Na⁺ and Cl[−], and the countertransport of one K⁺ molecule (Gu et al., 1994). This uptake process regulates the temporal and spatial actions of 5-HT on postsynaptic receptors and subsequent physiological processes such as mood, sleep, memory, appetite, and libido (Amara and Kuhar, 1993; Barker and Blakely, 1995).

In addition to regulating the downstream actions of 5-HT, SERT is the target of several structurally dissimilar compounds such as cocaine, amphetamines, tricyclic antidepressants (TCAs), and the selective serotonin reuptake inhibitors (SSRIs) (Owens et al., 1997; Amara and Sonders, 1998). Substrate translocation for the SERT and the related dopamine and norepinephrine transporters (DAT and NET) is thought to occur by an alternating access mechanism that exposes the permeation pathway successively to the extracellular and intracellular sections of the transporter (Zhang and Rudnick, 2006).

The third transmembrane helix (TMH) of SERT previously has been shown to contain residues that are associated with serotonin and cocaine binding (Chen et al., 1997b). Chen and colleagues used cysteine-scanning mutagenesis to demonstrate that two mutants, I172C and I179C were sensitive to inactivation by [2-(trimethylammonium)ethyl]methanethiosulfonate (MTSET) but were insensitive to (2-sulfonatoethyl)methanethiosulfonate (MTSES) (Chen et al., 1997b). Additionally, a cysteine substitution at residue Y176 completely blocked transport. Furthermore, mutants I172C and Y176C had reduced cocaine affinity, and Y176C had reduced 5-HT affinity in the presence of MTSET. The authors concluded that I172 and Y176 were not only on a helical face accessible to the aqueous environment, but were also involved in 5-HT and cocaine binding (Chen et al., 1997b).

Recent studies by Henry et al. (Henry et al., 2006) probed the contributions of TMH I and TMH III to antagonist recognition and determined that the human serotonin transporter mutant I172C displayed reductions in antagonist potencies, whereas substrate (5-HT, MDMA) recognition was not affected. Furthermore, the authors used engineered cysteine mutants to determine that an N101C/I179C double mutant was able to coordinate cadmium, providing evidence that some residues in TMH I and TMH III may be in proximity to each other. Studies by our group using engineered zinc-binding sites also have confirmed the proximity of hSERT TMH I and TMH III (White et al., 2006). Dose-response assays revealed that the double hSERT mutants V102C/M180C and V102C/I179C are more sensitive to zinc inhibition compared to the hSERT V102C single mutant (White et al., 2006). The increased sensitivity to zinc inhibition suggests that TMH I and TMH III are oriented in close proximity within the plasma membrane. A SERT homology model (Nichols, Walline, & Barker, unpublished data) generated from the coordinates of the *Aquifex aeolicus* leucine transporter structure (Yamashita et al., 2005) supports the proximity of TMH I and TMH III. Our model places residues N101 and I179 approximately 12 Å from each other. Additionally, the carbonyl oxygen of N101 is predicted to be within 3 Å of a sodium ion, and therefore, may be involved in sodium binding or 5-HT translocation. Furthermore, measured distances between V102C and I179C or M180C were less than 9 Å and were in general agreement with the experimental data (White et al., 2006).

We now extend our research on TMH III by employing species-scanning mutagenesis to probe the structure-activity relationships and molecular recognition of three distinct compound groups, the amphetamines, 3-phenyltropane analogs, and antidepressants. Comparative Molecular Field Analysis (CoMFA) was employed using selectivity fields that utilize the difference in pharmacological activities between the wild-type transporter and a point mutant as a dependent variable for structure-activity analysis (Baskin et al., 2003). This analysis allows visualization of the impact of mutations on ligand potency, providing new understanding for ligand recognition by SERT. To the best of our knowledge, this represents the first time selectivity field analysis has been used to discriminate the specific contribution of point mutations on ligand recognition. Additionally, selectivity field analysis may be useful as a unique tool for determining whether changes in drug potency at a mutant are due to specific protein-ligand interactions or disruption of binding sites due to conformational changes.

METHODS

Construction of TMH III mutants

The hSERT mutants I167L, A169D, F170I, I172M, and S174M were generated using the QuikChange® mutagenesis kit (Stratagene, La Jolla, CA) as per the manufacturer's instructions. The oligonucleotides used to generate the mutations were as follows: I167L-Sense: 5'-GGATTGGTTATGCCATATGCCTCATTGCC-3', I167L-Antisense: 5'-GGCAATGAGGCATATGGCATAACCAATCC-3', A169D-Sense: 5'-GGTTATGCCATATGCATCATTGACTTTTACATTGC-3', A169D-Antisense: 5'-GCAATGTAAAAGTCAATGATGCATATGGCATAACC-3', F170I-Sense: 5'-GGTTATGCCATATGCATCATTGCCATTTACATTGC-3', F170I-Antisense: 5'-GCAATGTAAATGGCAATGATGCATATGGCATAACC-3', I172M-Sense: 5'-GCCTTTACATGGCGTCCTACTACAACAAC-3', I172M-Antisense: 5'-GGTGTGTAGTAGGACGCCATGTAAAAGGC-3', S174M-Sense: 5'-GCCTTTACATTGCTATGTATTACAACACCATCATGGCC-3', S174M-Antisense: 5'-GGCCATGATGGTGTGTGTAATACATAGCAATGTAAAAGGC-3'. Mutant cDNAs were screened for a positive mutation using coding region silent restriction enzyme sites. Mutation-positive cDNAs were subcloned into the parental vector and the subcloned region was verified by sequencing (University of Michigan DNA Sequencing Core, Ann Arbor, MI).

5-HT transport assay—HeLa cells were grown in Dulbecco's modified Eagle's medium with 5% bovine calf serum and 5% Fetal Clone I and supplemented with 2 mM glutamine, and 1% penicillin/streptomycin. Cells were maintained in a humidified 5% CO₂ incubator at 37 °C. Cells were transfected using the vaccinia virus T7 (VVT₇) method using a 24- or 96-well format (Fuerst et al., 1986; Blakely et al., 1991; Roman et al., 2003). Briefly, HeLa cells were plated at a density of 1×10⁵ cells per well in 24-well culture plates (Perkin Elmer) or 2×10⁴ cells per well in 96-well plates 15–18 hours prior to transfection. Cells were infected with 10 pfu vaccinia virus per cell and transfected with 300 ng (24 well) or 100 ng (96 well) cDNA per well using the Lipofectin (Invitrogen) transfection method according to the manufacturer's protocol. Protein expression was allowed to increase during a 6–8 hour incubation. For [³H] 5-HT uptake assays, cells were washed once with 37 °C Krebs-Ringer-HEPES (KRH) buffer supplemented with calcium chloride and glucose (120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl₂, 10 mM HEPES, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1.8 g/l D-glucose, pH 7.4). Cells were preincubated for 10 min at 37 °C with increasing inhibitor concentrations. Transport assays with 20 nM [³H]5-HT, 100 μM L-ascorbic acid and 100 μM pargyline were performed for 10 min at 37 °C. Uptake was terminated by washing three times with ice-cold KRH. Non-specific uptake was determined using a vector transfection. Microscint 20 scintillation cocktail (Perkin Elmer) was added to each well and accumulated [³H]5-HT was determined on a PerkinElmer TopCount NXT. IC₅₀ values were determined with a non-linear regression analysis in GraphPad Prism version 3.0 (GraphPad Software Inc., San Diego, CA).

Kinetics assay—Saturation transport assays were performed using HeLa cells (1×10⁵ cells per well) in 24-well culture plates. At the time of assay, cells were washed once with KRH buffer. Cells were incubated with increasing concentrations of [³H]5-HT (final concentration ranged from 10 μM to 625 nM) with a specific activity diluted to ~0.1 Ci/mmol with unlabeled compound. Uptake was terminated exactly at 10 min by washing cells with ice-cold KRH buffer. Non-specific uptake was determined for each concentration using a vector transfection.

Amphetamine-induced efflux assay—HeLa cells were plated in 24-well plates and transfected as described above. Cells were incubated at 37 °C with [³H]5-HT (20 nM final concentration) for 10 min (hSERT) or 30 min (dSERT and hSERT A169D mutant) to provide for equivalent loading. Cells were treated with increasing concentrations of

dichloroamphetamine for 10 min and efflux was terminated by washing cells with ice-cold KRH buffer. The amount of [^3H]5-HT remaining in the cells was determined on a PerkinElmer TopCount NXT.

Basal efflux assay—HeLa cells were plated in 24-well plates and transfected as described above. Cells were washed once with 37 °C KRH. Next, [^3H]5-HT was added to each well at a final concentration of 40 nM. Radiolabeled substrate and cells were incubated for 30 min at 37 °C. Cells were washed two times with 37 °C and the second wash was left on for 2 min. Then, 250 μl 37 °C KRH was added to each well (except those used to define total accumulation) and aspirated after desired time. At the completion of the assay, all wells were washed one time with 800 μl ice-cold KRH.

Sodium dependency assay—HeLa cells were plated in 24-well plates and transfected as described above. At the time of assay, cells were washed once with sodium-free phosphate-buffered saline supplemented with calcium and magnesium (PBS-CM) (137 mM LiCl, 4.3 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 , 2.7 mM KCl, 0.1 mM CaCl_2 , and 1 mM MgCl_2 at pH 7.4). Cells were incubated in sodium-free PBS-CM isotonicly supplemented with increasing concentrations of NaCl and [^3H]5-HT (40 nM final concentration) at 37 °C for 10 min. Uptake was terminated by washing cells in ice-cold sodium-free PBS-CM. Non-specific uptake was determined using a vector transfected control.

Comparative Molecular Field Analysis (CoMFA) selectivity fields

Computational molecular modeling studies were performed using SYBYL version 7.1 software package (Tripos, St. Louis, MO) on a Silicon Graphics O2 workstation (Mountain View, CA) running IRIX version 6.5. The antidepressants, cocaine analogs, and amphetamines were sketched in SYBYL using library fragments. Atom types were manually checked and the active enantiomers of MDA, MDMA, fluoxetine, and citalopram were defined as *S*. *S*-fluoxetine was chosen as the potency is nearly identical to the *R* enantiomer and allowed for easy alignment with the other molecules. *1S*, *4S*-sertaline was used and included in the molecular modeling. The more active stereoisomer of tomoxetine was defined as *R* which is referred to in the text as the more common name atomoxetine. Each compound was subsequently minimized using the conjugate gradient minimization method with no initial simplex minimization, a gradient value of 0.05 kcal/mol, and 10,000 maximum iterations using MMFF94s force field parameters. A common substructure was drawn for the antidepressants using the two phenyl rings to align all of the minimized antidepressants. Three antidepressants, the SSRI fluvoxamine, the serotonin/norepinephrine reuptake inhibitor venlafaxine, and the serotonin transport blocker zimelidine were manually aligned to a phenyl ring, and selected centroid atoms in the remaining ring or chain because they lack two phenyl rings. Additionally, the side-chain torsions for clomipramine were defined based on the orientation observed bound in the crystal structure of the leucine transporter (Singh et al., 2007). The remaining antidepressant side chains were manually oriented to align to clomipramine so that the nitrogen atoms overlapped. Similarly, the tropane ring of cocaine was used to align cocaine and the RTI molecules (Research Triangle Institute, Research Triangle Park, NC). Additionally, the amphetamines were aligned to a phenethylamine template. One drug, dichlorophenylpiperazine was excluded from the CoMFA because the piperazine ring did not align well with the ethylamine side chain. A database for each drug class was created that contained the compound set as well as the substructure. Then, the pK_i values for the antidepressants, cocaine analogs, or amphetamines for each mutant were converted into selectivity field data using a modification of the equation as defined by Baskin and colleagues ($\log K_i(\text{hSERT mutant}) - (\log K_i(\text{wild-type hSERT}))$) (Baskin et al., 2003). The selectivity field data were inserted into an explicit data column in a molecular spreadsheet. Additionally, a CoMFA column was created using the Tripos Standard CoMFA field class, with steric and electrostatic

cutoffs at 30.0 kcal/mol. Molecular probe regions were manually defined with a minimum of 2 Å boundaries outside the molecule in the X, Y, and Z directions. CoMFA data were generated using 1–3 components based on the partial least squares analysis (PLS) for each molecule. Standard CoMFA contour specifications were used (80 for favored and 20 for disfavored) for steric and electrostatic contributions.

Data Analysis

The K_m and V_{max} in saturation experiments and IC_{50} values in transport assays were determined using non-linear curve-fitting analysis (Prism 3.0; GraphPad Software Inc., San Diego, CA). IC_{50} values were converted to K_i values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Statistical analyses of K_i values for [3H]5-HT uptake inhibition assays were performed with Prism using a one-way analysis of variance followed by a Dunnett's post-hoc test.

RESULTS

Transport kinetics of hSERT TMH III mutants

To investigate the role of TMH III in substrate and antagonist recognition, species-scanning mutagenesis was employed whereby the amino acid identity in *Drosophila* SERT (dSERT) was substituted into the equivalent position in hSERT. 5-HT uptake saturation analyses were then performed with hSERT and the TMH III mutants. These results indicated that the 5-HT transport velocity for the F170I and I172M mutants did not differ significantly from WT hSERT (Table 1). Maximal transport capacity for the I167L mutant was low (<5% of wild-type), thus, limiting our ability to reliably determine transport kinetic parameters. This resulted from limitations associated with detection in the [3H]5-HT uptake assays. Interestingly, the S174M mutant had a statistically significant increase in maximal 5-HT transport and the A169D mutant had a statistically significant decrease in maximal 5-HT transport (hSERT, $23 \pm 7 \times 10^{-17}$ mol/cell/min; S174M, $180 \pm 60 \times 10^{-17}$ mol/cell/min; A169D, 1.6 ± 0.4 mol/cell/min). The S174M and F170I mutants had an increased K_m for 5-HT transport, indicating a possible decrease in transporter affinity for 5-HT. In contrast, the I172M mutant had a decreased K_m for 5-HT transport, suggesting an increase in transporter affinity for 5-HT.

Recognition of amphetamines by hSERT mutants

The potencies of the amphetamines were evaluated at the hSERT mutants I167L, A169D, F170I, I172M, and S174M (Table 2). For the hSERT F170I mutant, there were no statistically significant changes in amphetamine potency. For the hSERT I167L mutant, only 3,4-methylenedioxy-2-(*N,N*-dipropylamino)indan (*N*-Dipropylindan) and AMMT had significantly increased potency at the hSERT I167L mutant. The remaining mutants, however, exhibited differences in potencies for several of the amphetamines.

Selectivity field CoMFAs were performed on the hSERT A169D mutant (Baskin et al., 2003). Selectivity fields allow the differences in biological activity between a wild-type transporter and a mutant transporter to be visualized as colored CoMFA fields. The fields generated from explicit data were expressed as the difference between activity of the wild-type and mutant transporters using the following equation: $(\log K_i \text{ (hSERT mutant)}) - (\log K_i \text{ (wild-type hSERT)})$. The hSERT A169D mutant showed a significant increase in potency for ten amphetamines (DFA, MDMA, DiFMDA, 2MeMDA, 4-TFMA, AMMT, AMMI, DCPD, MM(R)NMA, and *N*-dipropylindan (Table 2). The selectivity field CoMFA for the A169D mutant, however, showed no predominating fields, indicating that the increase in potency of the amphetamine analogs is probably not a direct result of hydrogen bonding or steric interactions, but rather may result from a global structural change in the SERT protein (Figure 1).

The I172M and S174M mutants showed statistically significant reductions in potency for six and fourteen of the amphetamine analogues, respectively (Table 2). Once more, the selectivity field-based CoMFA showed no specific contribution of the amphetamine structure to potency for the I172M mutant (Figure 2). Therefore, changes in amphetamine potency at the A169D and I172M mutants also may be due to conformation changes in tertiary structure resulting from the point mutations.

Decreases in potency for uptake competition by the amphetamines at the S174M mutant may be attenuated by addition of negative charge or hydrogen bond acceptors to the 4-position of the phenyl ring as well as the nitrogen side chain (Figure 3). This mutation might eliminate an important hydrogen-bond interaction that decreases drug potency.

Mutation-induced effects of substrate efflux

Previous work by our group has demonstrated that amphetamines were poorly transported by and unable to induce substrate efflux at the dSERT (Rodriguez et al., 2003). Of the five hSERT mutants constructed with a dSERT residue, only the A169D mutant gained a net negative charge. We hypothesized that introduction of the charged aspartate may disrupt the translocation of positively charged amphetamines, leading to decreased amphetamine-induced efflux in the case of dSERT. Substrates such as *p*-chloroamphetamine (PCA) and methylenedioxymethamphetamine (MDMA) promote a reversed transport mechanism in hSERT that can be used to explore the mechanism of the bidirectional 5-HT translocation process (Sitte et al., 2000). Carrier-mediated efflux for hSERT, dSERT, and the hSERT A169D mutant was assessed using dichloroamphetamine (DCA) to determine the potency of these drugs at inducing efflux. DCA produced a modest efflux of 5-HT from hSERT (Figure 4A). However, concentrations of DCA up to 10 μ M were not able to induce an appreciable efflux from the hSERT A169D mutant or dSERT (Figure 4B). Indeed, for A169D, efflux of preloaded [3 H]5-HT occurred in the absence of drug, indicating a possible alteration in the 5-HT translocation cycle. Therefore, the basal efflux rate for hSERT, the A169D mutant, and dSERT was determined. The initial rate of loss of radiolabeled 5-HT from preloaded cells was determined from remaining accumulated 5-HT at 0, 5, 10, and 15 seconds (Figure 4C). When total transport is normalized to 100 percent, dSERT and the A169D mutant efflux a higher percentage of preloaded 5-HT over time (Figure 4C). Interestingly, the A169D mutant and dSERT both retained only 75% of transported 5-HT after 15 sec as compared with 95% for hSERT (Figure 4C). The actual rates of basal [3 H]5-HT efflux in the presence of KRH buffer for hSERT, the A169D mutant, and dSERT were $25 \pm 5 \times 10^{-21}$ mol/cell/min, $0.8 \pm 0.1 \times 10^{-21}$ mol/cell/min, and $1.5 \pm 0.7 \times 10^{-21}$ mol/cell/min, respectively. The rate of spontaneous efflux for WT hSERT is statistically significantly greater than for either the A169D mutant or dSERT ($p < 0.05$, one-way ANOVA, Bonferroni's post-hoc test), and the rates for the A169D mutant and dSERT do not differ from each other. This greater hSERT efflux rate is influenced, however, by the increased overall number of 5-HT molecules transported by hSERT as compared to dSERT and the hSERT A169D mutant. These results indicate that by substituting the aspartate present in dSERT for the corresponding alanine at position 169 in hSERT, the serotonin efflux process is altered and basal efflux resembles that of dSERT. This finding may indicate that A169D is involved in a substrate exchange mechanism, as our SERT homology model places residue 169 near the Na⁺ binding site and near the cytoplasmic interface for substrate translocation.

Sodium dependency assay and transport competition assay with MDMA

We constructed an hSERT homology model from the LeuT_{Aa} crystal structure, a bacterial transporter and orthologue to the monoamine transporters (Yamashita et al., 2005). The model was based on a manual sequence alignment of the leucine transporter and SERT (Nichols, Walline, & Barker, unpublished data). Our homology model revealed that the A169 residue is

in the same plane of the transporter as the two hypothesized sodium ions necessary for leucine transport (Yamashita et al., 2005). We hypothesized that the gain in potency for many of the amphetamine analogues in the A169D mutant could be due to an alteration in sodium requirements. We determined that the sodium dependence for the A169D mutant was not saturable over the concentration of sodium used in our experiments (Fig. 5A). In contrast, hSERT displayed one-site binding kinetics and had a K_m value of 40 mM (Figure 5B). Our results indicate that the hSERT A169D mutant may have reduced affinity for sodium or a more complex interaction with the cation similar to the sodium requirements for the DAT and NET (Gu et al., 1994).

Since the A169D mutant exhibits altered sodium dependence, we speculated that the increase in potency of MDMA and other amphetamines at the A169D mutant could be impacted by changes in sodium concentration. If this were true, these potency differences might be abolished in the presence of decreased sodium concentrations. Therefore, 5-HT competition assays were performed for hSERT and the A169D mutant in KRH buffer containing 25 mM NaCl or 150 mM NaCl. Similar to previous observations, the potency of MDMA to block 5-HT transport at the hSERT A169D mutant was greater than at wild-type SERT in the presence of 150 mM NaCl (A169D, $1.3 \pm 0.2 \mu\text{M}$ vs. hSERT, $6.1 \pm 1 \mu\text{M}$, $p < 0.01$ using a Student's t-test). Nevertheless, increased potency of MDMA at the hSERT A169D mutant also was observed in the presence of only 25 mM NaCl (A169D, $0.8 \pm 0.08 \mu\text{M}$ vs. hSERT, $5.1 \pm 0.5 \mu\text{M}$, $p < 0.05$ using a Student's t-test)(data not shown).

Recognition of cocaine (3-phenyltropane) analogs by hSERT mutants

3-phenyltropane analogs were used to evaluate antagonist inhibition potency for the TMH III mutants. Only one construct, hSERT I172M, showed a reduction in antagonist potency for the cocaine analogs (see Table 3). All analogs except RTI-55 and RTI-121 exhibited reduced potency compared to the wild-type SERT. Both RTI-55 and RTI-121 contain an iodine substitution in the 4-position on the phenyl ring. The presence of the electronegative iodine atom will make the phenyl ring slightly more positive relative to an unsubstituted phenyl ring. The selectivity field CoMFA indicated that positive charge around the phenyl ring increases the potency of the cocaine analogs at the I172M mutant (Figure 6). Indeed, our data for RTI-55 and RTI-121 support this model. Therefore, areas of positive charge and/or hydrogen bond donation may contribute to a high potency interaction of the cocaine analogs when Met substitutes for Ile at position 172. The predominant red selectivity field suggests this interaction could be a direct contact site for the cocaine analogs. In our homology model based on the leucine transporter, I172 lies directly below the Glu493 – Arg104 “gate”, and lies along the likely permeation pathway. Although I172 can interact with local aromatic residues through van der Waals interactions, M172 also can interact with aromatic rings through a sulfur- π complex (Tatko and Waters, 2004). Further, I172 is a rather compact residue compared to Met, whereas methionine projects far out into the putative permeation pathway. Thus, we hypothesize that the non-halogenated cocaine analogues do not bind as well in the I172M mutant because of the steric projection of M172 into the permeation pathway, impeding access to an area in the transporter that may be involved in cocaine binding. By contrast, the iodinated cocaine analogues have increased affinity to the M172 mutation because they have the ability to form a complex between their more positive aromatic ring and the polarizable sulfur atom of the methionine that may compensate for the larger steric size of the methionine residue. This reasoning is compatible with our results suggesting that I172 may be a contact site for cocaine binding.

Recognition of antidepressants by hSERT mutants

Twenty antidepressants were screened for uptake inhibition potency at the TMH III mutants (Table 4). No statistically significant changes of antagonist potency were seen for the I167L,

A169D, F170I, or S174M mutants. In contrast, statistically significant decreases in antagonist potency at the I172M mutant were seen for all of the antidepressants except for amoxepine, maprotiline, and 3 α -bis-(4-fluorophenyl)-methoxytropine, a potent dopamine transporter inhibitor (Newman et al., 1994). The selectivity field CoMFA of the antidepressant data showed no one predominating colored field indicating that the loss of potencies for the antidepressant set may be the result of a global conformational change and not due to disruption of a direct binding site (Figure 7).

Alternatively, CoMFAs assume that all compounds in the test set are binding in the same location within a biological target and share a common core structure. The broad structural diversity associated with the core chemical structures of the antidepressant compound set may not be ideal for CoMFA selectivity field analysis, as the antidepressants may be binding to multiple sites within SERT, thus confounding the analysis and resulting in the presence of multiple fields.

DISCUSSION

Considerable attention has been given to studying the third transmembrane helix in monoamine transporters. Previously, Bismuth and colleagues determined that the γ -aminobutyric acid (GABA) transporter (GAT) residue Y140, a residue conserved in the third TMH throughout the sodium/chloride-dependent transporter family, is critical for GABA recognition and transport (Bismuth et al., 1997). Furthermore, residue Y289 of the neuronal glycine transporter GLYT2 has an important role in glycine binding and transport, ion dependence, and sodium selectivity (Ponce et al., 2000). GAT Y140 and GLYT2 Y289 are both homologous to Y176 in the hSERT. hSERT Y176 as well as I172C and I179C were shown to be sensitive to inactivation by MTSET (Chen et al., 1997b). Residues I172, Y176, and I179 were suggested to face towards the inside of the pore, and residue I172 was proposed to reside close to the substrate binding site (Chen et al., 1997a; Chen et al., 1997b). An hSERT homology model constructed from the crystal structure of a prokaryotic amino acid transporter from *Aquifex aeolicus* (LeuT_{Aa}) supports these experimental data and places residues I172, Y176, and I179 facing the putative substrate binding site (Beuming et al., 2006). Therefore, the third transmembrane domain of GAT, GLYT2, and SERT each contain residues critical for substrate recognition, sodium binding, and antagonist binding, supporting the hypothesis that THM III is part of the permeation pathway and is critical for substrate translocation and antagonist recognition.

Our group used species-scanning mutagenesis to generate five hSERT TMH III mutants where the amino acid identity in dSERT was substituted into the equivalent position in hSERT. The potencies of the amphetamines, 3-phenyltropane analogs, and antidepressants were evaluated at the hSERT mutants. Subsequently, CoMFAs were performed using a modification of the approach developed by Baskin and colleagues, where the difference in pharmacological activity between the WT and mutant transporter is expressed as a dependent variable in the CoMFA spreadsheet. For our purposes, the pharmacological activity of the wild-type hSERT was subtracted from the pharmacological activity of the mutant to determine the role of the point mutation with respect to antagonist or substrate potency (Baskin et al., 2003). Therefore, the colored contour fields are a visual representation of the effect of the mutation on amphetamine, cocaine, or antidepressant interaction with the SERT protein. The resulting fields directly illustrate the steric or electrostatic interactions induced by the mutation that alter drug potency. Traditional CoMFAs require visual inspection of two separate contour maps to determine differences in pharmacological activity between two proteins, with comparisons performed in a qualitative manner. The use of CoMFA selectivity fields semi-quantitatively represents the change in drug potency between two proteins. The size of the selectivity field

can be directly observed and eliminates the need to visually compare differences in field size for two separate analyses.

Our results from the uptake competition assays revealed significant decreases in transport inhibition potencies for nearly all the amphetamines at the S174M mutant. In the LeuT_{Aa}, S174 corresponds to A105. A105, V103, I106, and M403 participate in a hydrophobic cluster that would stabilize the helical packing forces between TMHs I and X. By contrast, in the SERT, S174 appears to participate in a hydrogen-bonded network in a homologous region that includes Y171, and K490. Again, this network would stabilize packing between helices III and X. Mutating residue S174 would disrupt this interaction, loosening the association between helix III and helix X. Helix X is directly adjacent to the critical R104, and any change in the relative conformation of TMH X is likely to affect function, thus potentially disrupting the ability of the amphetamines to inhibit transport. The selectivity field CoMFA indicates that alterations in hydrogen bonding or charges in various positions around the amphetamine structure could be responsible for changes in drug potency. It is possible that by replacing the one-carbon hydroxy-substituted serine with a three-carbon thioether containing methionine that critical hydrogen bonding or electrostatic interactions could have been disrupted. Alternatively, a narrow green field is shown wrapping around the amphetamine structure, which suggests that introduction of the methionine residue leads to a transporter that disfavors bulky amphetamines. The presence of multiple, small fields suggests that changes in drug potency likely result from a global change in protein structure and are not representative of a direct contact site for the amphetamines at residue S174.

Six of the amphetamine analogs tested (DiFMDA, MDA, 2MeMDA, 6MeMDA, AEPCA, and AEMMA) showed significant decreases in potency for transport inhibition at the I172M mutant. The selectivity field CoMFA shows several colored fields, indicating that this mutation causes a disruption in antagonist recognition and may indicate a global conformational change of the SERT protein.

Additional selectivity field CoMFAs were performed for the cocaine analogs and the antidepressants at the I172M mutant. The cocaine analog analysis indicated that positive charge around the phenyl ring increases the relative potency of the drug for the I172M mutant. The presence of one large field for the cocaine analog analysis indicates that this position may represent a direct contact site for the cocaine analogs (Figure 6). This result supports previous research suggesting that I172 and Y176 are in proximity to the 5-HT and cocaine binding site (Chen et al., 1997b).

Analysis of the antidepressants provided several selectivity fields, however, which suggest that the I172M mutation may cause a global disruption in antidepressant recognition by influencing the conformation associated with nearby residues that may be involved in directly interacting with the antidepressants (Figure 7). Furthermore, the fact that the 3-phenyltropane analogs revealed evidence consistent with a direct interaction suggests distinct, but possibly overlapping sites for the different classes of drugs. Alternatively, the differences found in the core chemical structure of our antidepressant compound set did not allow for a molecular alignment in the CoMFA that was as good as that for the 3-phenyltropanes or the amphetamines. This poor alignment may have masked a direct interaction with one or more antidepressants. Earlier studies implicated residues I172 and Y95 as determinants of citalopram potency (Henry et al., 2006). The residue homologous to I172 in the LeuT_{Aa} structure projects into a hydrophobic binding pocket to accommodate the hydrophobic methyl groups of the substrate (Yamashita et al., 2005). Additionally, cocaine analogs are thought to bind in a highly hydrophobic microenvironment (Rasmussen et al., 2001). Altogether our results support the involvement of I172 with cocaine recognition, but not in the binding of any of the antidepressants.

Two very recent reports have described a TCA binding site within the LeuT_{Aa} structure (Singh et al., 2007; Zhou et al., 2007). Both groups identified residues extracellular to the gating residues R104 and E493 as contact sites for the TCAs. Based on our hSERT homology model, these putative TCA binding site residues range from 10–22 Å from the TMH III residue I172. The selectivity field analysis indicates that hSERT mutant I172M is not likely to be a direct contact site for the antidepressant compound set.

Our group has previously shown that substituted amphetamines are not effectively transported by dSERT, and therefore have decreased efficacy for inducing 5-HT efflux (Rodriguez et al., 2003). Additionally, unlabeled 5-HT was less capable of inducing substrate efflux. It is possible that the aspartate in the A169D mutant and wild-type dSERT may be disrupting the release of substrate, resulting in a loss of amphetamine-induced efflux (Figure 4B). A lack of substrate-induced efflux is characteristic of channels and may indicate that the A169D mutant and dSERT are operating in a channel-like mode (Rodriguez et al., 2003). This finding may suggest that the aspartate in position 169 is involved with gating SERT channel-like properties.

Sodium dependency assays indicated that the hSERT A169D mutant was not saturable over the range of sodium concentrations used in our studies. We believe that this altered sodium dependence is most likely the result of an increase in the K_m value for sodium at the A169D mutant. This reduced affinity for sodium could disrupt 5-HT transport and this alteration in uptake capacity may be overcome in the presence of higher sodium concentrations (Figure 5). Interestingly, the potency of MDMA did not change with differing sodium concentrations. Therefore, the sodium requirements for the hSERT A169D mutant may perturb 5-HT transport without affecting the potency of other substrates.

In conclusion, our results indicate that hSERT transmembrane helix III likely contributes to the permeation pathway and that residues I172 and A169 could represent important contact sites for antagonist recognition and substrate transport. The selectivity field CoMFAs utilized the difference in biological activities between hSERT and selected point mutants to visualize the impact of changes in activity. This analysis is a useful tool to distinguish between general conformational changes and potential direct protein-ligand interactions. Additionally, the hSERT mutant A169D had altered sodium requirements, suggesting that this residue is near the sodium-coordinating site. This result is consistent with our homology model, which places the aspartate less than 10 Å from a Na⁺ binding site in LeuT_{Aa}. Thus, our study has used a novel computational method to integrate mutagenesis and structure-activity relationship data to explore hSERT TMH III as an important domain for substrate selectivity and antagonist binding.

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Nonstandard abbreviations

5-HT, 5-hydroxytryptamine, serotonin
SERT, serotonin transporter
CoMFA, comparative molecular field analysis
TMH, transmembrane helix
KRH, Krebs-Ringer-HEPES
PBS-CM, phosphate buffered saline substituted with calcium and magnesium
PLS, partial least-squares

DAT, dopamine transporter
 NET, norepinephrine transporter
 SSRI, selective serotonin reuptake inhibitor
 TCA, tricyclic antidepressant
 CPT, CPT-D-tartrate, (-)-2 β -carbomethoxy-3 β -phenyltropane tartrate
 RTI-31, 3 β -(4-chlorophenyl)tropane-2 β -carboxylic acid methyl ester tartrate
 RTI-32, 3 β -(4-methylphenyl)tropane-2 β -carboxylic acid methyl ester tartrate
 RTI-55, 3 β -(4-iodophenyl)tropane-2 β -carboxylic acid methyl ester tartrate
 RTI-83, 3 β -(4-ethylphenyl)tropane-2 β -carboxylic acid methyl ester tartrate
 RTI-112, (-)-3 β -(3-methyl-4-chlorophenyl)tropane-2 β -carboxylic acid methyl ester tartrate
 RTI-121, 3 β -(4-iodophenyl)-tropane-2 β -carboxylic acid isopropyl ester hydrochloride
 RTI-142, (-)-*N*-nor-3 β -(4-fluorophenyl)tropane-2 β -carboxylic acid methyl ester
 RTI-147, 3 β -(4-chlorophenyl)tropane-2 β -pyrrolidinecarboxamide hydrochloride
 RTI-311, *N*-allyl-*N*-nor-3 β -(4-iodophenyl)tropane-2 β -carboxylic acid methyl ester tartrate
 DCA, 3,4-dichloroamphetamine
 4-MTA, 4-methylthioamphetamine
 3-MTA, 3-methylthioamphetamine
 2-Me-MDA, 2-methyl-3,4-methylenedioxyamphetamine
 5-Me-MDA, 5-methyl-3,4-methylenedioxyamphetamine
 6-Me-MDA, 6-methyl-3,4-methylenedioxyamphetamine
 MMAI, 5-methoxy-6-methyl-2-aminoindan
 MDA, 3,4-methylenedioxyamphetamine
 MDMA, 3,4-methylenedioxy-*N*-methyl-amphetamine
 AMMI, 1-aminomethyl-5-methoxyindan
 DFA, 3,4-difluoroamphetamine
 4-TFMA, 4-trifluoromethylamphetamine
 AMMT, 1-aminomethyl-6-methoxytetralin
 MSA, 4-methylsulfonylamphetamine
N-dipropyl-indan, 3,4-methylenedioxy-2-*N*-*N*-dipropylindan
 AEMMA, 3-methoxy-4-methyl- α -ethylphenethylamine
 DCPD, 3,4-dichlorophenylpiperazine
 DiFMDA, 1-(2,2-difluorobenzo[*d*][1,3]dioxol-5-yl)propan-2-amine
 MM(S)NMA, (*S*)-3-methoxy-4-methyl-*N*-methyl-amphetamine
 MM(R)NMA, (*R*)-3-methoxy-4-methyl-*N*-methyl-amphetamine
 3- α -bis, 3- α -bis-(4-fluorophenyl)methoxytropane hydrochloride

REFERENCES

- Amara SG, Kuhar MJ. Neurotransmitter transporters: recent progress. *Annu.Rev.Neurosci* 1993;16:73–93. [PubMed: 8096377]
- Amara SG, Sonders MS. Neurotransmitter transporters as molecular targets for addictive drugs. *Drug Alcohol Depend* 1998;51:87–96. [PubMed: 9716932]
- Barker, EL.; Blakely, RD. Norepinephrine and Serotonin Transporters: Molecular Targets of Antidepressants Drugs. In: Bloom, FE.; Kupfer, DJ., editors. *Pharmacology: The Fourth Generation of Progress*. Raven Press; New York: 1995. p. 321-333.
- Baskin II, Tikhonova IG, Palyulin VA, Zefirov NS. Selectivity fields: comparative molecular field analysis (CoMFA) of the glycine/NMDA and AMPA receptors. *J.Med.Chem* 2003;46:4063–4069. [PubMed: 12954059]
- Beuming T, Shi L, Javitch JA, Weinstein H. A Comprehensive Structure-Based Alignment of Prokaryotic and Eukaryotic Neurotransmitter/Na⁺ Symporters (NSS) Aids in the Use of the LeuT Structure to Probe NSS Structure and Function. *Mol.Pharmacol* 2006;70:1630–1642. [PubMed: 16880288]

- Bismuth Y, Kavanaugh MP, Kanner BI. Tyrosine 140 of the gamma-aminobutyric acid transporter GAT-1 plays a critical role in neurotransmitter recognition. *J.Biol.Chem* 1997;272:16096–16102. [PubMed: 9195904]
- Blakely RD, Clark JA, Rudnick G, Amara SG. Vaccinia-T7 RNA polymerase expression system: evaluation for the expression cloning of plasma membrane transporters. *Anal.Biochem* 1991;194:302–308. [PubMed: 1862934]
- Chen JG, Liu-Chen S, Rudnick G. External cysteine residues in the serotonin transporter. *Biochemistry* 1997a;36:1479–1486. [PubMed: 9063896]
- Chen JG, Sachpatzidis A, Rudnick G. The third transmembrane domain of the serotonin transporter contains residues associated with substrate and cocaine binding. *J.Biol.Chem* 1997b;272:28321–28327. [PubMed: 9353288]
- Cheng Y, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem.Pharmacol* 1973;22:3099–3108. [PubMed: 4202581]
- Fuerst TR, Niles EG, Studier FW, Moss B. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc.Natl.Acad.Sci.U.S.A* 1986;83:8122–8126. [PubMed: 3095828]
- Gu H, Wall SC, Rudnick G. Stable expression of biogenic amine transporters reveals differences in inhibitor sensitivity, kinetics, and ion dependence. *J.Biol.Chem* 1994;269:7124–7130. [PubMed: 8125921]
- Henry LK, Field JR, Adkins EM, Parnas ML, Vaughan RA, Zou MF, Newman AH, Blakely RD. Tyr-95 and ile-172 in transmembrane segments 1 and 3 of human serotonin transporters interact to establish high affinity recognition of antidepressants. *J.Biol.Chem* 2006;281:2012–2023. [PubMed: 16272152]
- Newman AH, Allen AC, Izenwasser S, Katz JL. Novel 3 alpha-(diphenylmethoxy)tropane analogs: potent dopamine uptake inhibitors without cocaine-like behavioral profiles. *J.Med.Chem* 1994;37:2258–2261. [PubMed: 8057273]
- Owens MJ, Morgan WN, Plott SJ, Nemeroff CB. Neurotransmitter receptor and transporter binding profile of antidepressants and their metabolites. *J.Pharmacol.Exp.Ther* 1997;283:1305–1322. [PubMed: 9400006]
- Ponce J, Biton B, Benavides J, Avenet P, Aragon C. Transmembrane domain III plays an important role in ion binding and permeation in the glycine transporter GLYT2. *J.Biol.Chem* 2000;275:13856–13862. [PubMed: 10788509]
- Rasmussen SG, Carroll FI, Maresch MJ, Jensen AD, Tate CG, Gether U. Biophysical characterization of the cocaine binding pocket in the serotonin transporter using a fluorescent cocaine analogue as a molecular reporter. *J.Biol.Chem* 2001;276:4717–4723. [PubMed: 11062247]
- Rodriguez GJ, Roman DL, White KJ, Nichols DE, Barker EL. Distinct recognition of substrates by the human and *Drosophila* serotonin transporters. *J.Pharmacol.Exp.Ther* 2003;306:338–346. [PubMed: 12682215]
- Roman DL, Walline CC, Rodriguez GJ, Barker EL. Interactions of antidepressants with the serotonin transporter: a contemporary molecular analysis. *Eur.J.Pharmacol* 2003;479:53–63. [PubMed: 14612137]
- Singh SK, Yamashita A, Gouaux E. Antidepressant binding site in a bacterial homologue of neurotransmitter transporters. *Nature* 2007;448:952–956. [PubMed: 17687333]
- Sitte HH, Scholze P, Schloss P, Pifl C, Singer EA. Characterization of carrier-mediated efflux in human embryonic kidney 293 cells stably expressing the rat serotonin transporter: a superfusion study. *J.Neurochem* 2000;74:1317–1324. [PubMed: 10693966]
- Tatko CD, Waters ML. Investigation of the nature of the methionine- π interaction in β -hairpin model systems. *Protein Sci* 2004;13:2515–2522. [PubMed: 15322289]
- White KJ, Kiser PD, Nichols DE, Barker EL. Engineered zinc-binding sites confirm proximity and orientation of transmembrane helices I and III in the human serotonin transporter. *Protein Sci* 2006;15:2411–2422. [PubMed: 17008722]
- Yamashita A, Singh SK, Kawate T, Jin Y, Gouaux E. Crystal structure of a bacterial homologue of Na⁺/Cl⁻-dependent neurotransmitter transporters. *Nature* 2005;437:215–223. [PubMed: 16041361]

- Zhang YW, Rudnick G. The cytoplasmic substrate permeation pathway of serotonin transporter. *J.Biol.Chem* 2006;281:36213–36220. [PubMed: 17008313]
- Zhou Z, Zhen J, Karpowich NK, Goetz RM, Law CJ, Reith ME, Wang DN. LeuT-desipramine structure reveals how antidepressants block neurotransmitter reuptake. *Science* 2007;317:1390–1393. [PubMed: 17690258]

Figure 1. Selectivity field map of amphetamine structure-activity relationship for hSERT mutant A169D. The presence of a yellow field indicates that steric bulk is disfavored at the mutant as compared to the wild-type hSERT. In contrast, the presence of a green field indicates that steric bulk in this region is favored for drug potency at the mutant. A blue field indicates that negative charge and/or H-bond acceptors in the ligand are favored for increased potency at the mutant, whereas a red field represents regions where increased positive charge and/or H-bond donors in the ligand are favored for increased potency. The structure of dichloroamphetamine is shown within the field for reference. The selectivity field map for the hSERT A169D mutant reveals no predominant colored fields, suggesting that the observed increase in potency of several amphetamines for the A169D mutant is most probably due to global structural changes within the SERT protein.

Figure 2. Selectivity field map of amphetamine structure activity relationship for hSERT mutant I172M. The reference molecule and the interpretation of the colored selectivity field maps are described in Figure 1. The selectivity field map for the hSERT I172M mutant reveals no predominant colored fields, again suggesting that the observed decrease in potency of several amphetamines for the I172M mutant is due to overall tertiary structural changes within the SERT protein.

Figure 3. Selectivity field map of amphetamine structure activity relationship for hSERT mutant S174M. The reference molecule and colored selectivity field maps are described in Figure 1. The selectivity field map for the hSERT S174M mutant reveals a blue field around the 4-position of the phenyl ring and the nitrogen side chain. Addition of negative charge and/or H-bond acceptors to these regions of the ligand is favored for increased potency at this mutant. The presence of a green field suggests that steric bulk is favored for potency at the mutant.

Figure 4.

Dichloroamphetamine-induced efflux in hSERT, dSERT, and the hSERT A169D mutant. Cells were preloaded with 20 nM [³H]5-HT. Cells were washed and treated with increasing concentrations of DCA. Data is shown as percent specific 5-HT remaining. DCA promotes efflux from hSERT (A) but not dSERT or the hSERT A169D mutant (B). Basal [³H]5-HT efflux from hSERT, dSERT, and the hSERT A169D mutant in the presence of KRH buffer (C). Efflux assays were performed on transiently transfected HeLa cells, as described under Materials and Methods. Total transport is normalized to 100% for each construct and the data represent % transported [³H]5-HT remaining vs. time. Data shown are representative means $\pm$ S.E. of three separate experiments performed in triplicate.

Figure 5.

Effect of $[\text{Na}^+]$ on $[^3\text{H}]5\text{-HT}$ transport for hSERT and the A169D mutant. Assays were performed on transiently transfected HeLa cells, as described in Materials and Methods. $[^3\text{H}]5\text{-HT}$ uptake was determined for the hSERT A169D mutant (A) and wild-type hSERT (B) in the presence of KRH containing increasing concentrations of Na^+ . The buffer was supplemented with LiCl to maintain ionic strength. Data for the hSERT A169D mutant was fit to both one-site and two-site binding curves. An F-test comparison of the two fits did not support the use of the two-site fit (F ratio=0.72; $P=0.5$), thus, the one-site fit is shown although the data indicate that saturation has not been achieved. Data for hSERT were fit to a one-site binding non-linear regression analysis. The Na^+ K_m value for hSERT is 40 mM. Na^+ K_m value

for the A169D mutant was estimated to be greater than 150 mM. Data shown are representative means $\pm$ S.E. of three separate experiments performed in triplicate.

Figure 6. Selectivity field map of 3-phenyltropane analog structure activity relationship for hSERT mutant I172M. The structure of RTI-55 is shown within the field for reference. The interpretation of the colored selectivity field maps is described in Figure 1. The presence of red field around the phenyl ring suggests that increased positive charge of the aromatic ring increases potency of the 3-phenyltropane analog at this mutant.

Figure 7. Selectivity field map of antidepressant structure activity relationship for hSERT mutant I172M. The structure of clomipramine is shown within the field for reference. The interpretation of the colored selectivity field maps is described in Figure 1. The selectivity field map for the hSERT I172M mutant reveals no predominant colored fields, suggesting that the observed decrease in potency for most of the antidepressants for the I172M mutant is due to overall tertiary structural changes within the SERT protein.

Table 1

Saturation uptake analysis of hSERT and TMH III mutants for 5-HT transport. HeLa cells transiently expressing the wild-type or mutant transporters were incubated with radiolabeled 5-HT as described in Materials & Methods. Data represent the mean $\pm$ SEM for at least three independent experiments performed in triplicate. Statistical significance was determined using an ANOVA (*) followed by a Dunnett's post-hoc test or a Student's t-test ($\dagger$).

	$V_{\max}$ (mol/cell/min $\times 10^{-17}$)	K_m (μ M)
hSERT	23 ± 7	0.9 ± 0.2
I167L	N.D.	N.D.
A169D	$1.6 \pm 0.4^{\dagger}$	1.7 ± 0.8
F170I	16 ± 8	$5.3 \pm 3.0^{*\dagger}$
I172M	27 ± 3	$0.2 \pm 0.02^{\dagger}$
S174M	$180 \pm 60^{*\dagger}$	$3.8 \pm 1.0^{*\dagger}$

* $p < 0.05$ vs. hSERT

$\dagger$ $p < 0.05$ vs. hSERT; (N.D.) not determined.

Table 2

Substituted amphetamine K_i values (nM) for [3 H]5-HT uptake inhibition at hSERT, and the TMH III mutants, I167L, A169D, F170I, I172M, S174M. [3 H]5-HT uptake assays were performed on transiently transfected HeLa cells, as described in Materials and Methods. Data represent the mean $\pm$ SEM for at least three independent experiments performed in triplicate.

	hSERT	I167L	A169D	F170I	I172M	S174M
DCA	130 $\pm$ 10	40 $\pm$ 10	20 $\pm$ 4	90 $\pm$ 40	240 $\pm$ 70	700 $\pm$ 20 ^{***}
DFA	5600 $\pm$ 200	3900 $\pm$ 80	400 $\pm$ 200 ^{***}	3700 $\pm$ 1400	2800 $\pm$ 70	11000 $\pm$ 700 ^{***}
MDA	700 $\pm$ 100	500 $\pm$ 200	200 $\pm$ 10	500 $\pm$ 10	2600 $\pm$ 600 ^{***}	2400 $\pm$ 300 ^{***}
MDMA	1600 $\pm$ 500	300 $\pm$ 70	200 $\pm$ 80 [*]	600 $\pm$ 90	2000 $\pm$ 500	3700 $\pm$ 200 ^{***}
DiFMDA	1200 $\pm$ 200	900 $\pm$ 200	200 $\pm$ 40 ^{***}	1100 $\pm$ 50	2400 $\pm$ 300 ^{***}	2600 $\pm$ 100 ^{***}
MSA	53000 $\pm$ 17000	21000 $\pm$ 1400	12000 $\pm$ 300	19000 $\pm$ 5400	51000 $\pm$ 2600	160000 $\pm$ 21000 ^{***}
2MeMDA	700 $\pm$ 200	400 $\pm$ 60	200 $\pm$ 60 [*]	400 $\pm$ 100	1300 $\pm$ 30 ^{***}	1000 $\pm$ 80
5MeMDA	900 $\pm$ 70	500 $\pm$ 90	600 $\pm$ 40	600 $\pm$ 80	1300 $\pm$ 500	3000 $\pm$ 500 ^{***}
6MeMDA	2200 $\pm$ 600	1400 $\pm$ 500	3100 $\pm$ 800	2200 $\pm$ 700	10000 $\pm$ 600 ^{***}	4500 $\pm$ 900
3-MTA	2500 $\pm$ 500	1500 $\pm$ 300	300 $\pm$ 60	1100 $\pm$ 300	2200 $\pm$ 500	4900 $\pm$ 1700
4-MTA	200 $\pm$ 20	300 $\pm$ 70	200 $\pm$ 30	200 $\pm$ 20	200 $\pm$ 50	800 $\pm$ 80 ^{***}
4-TFMA	600 $\pm$ 70	300 $\pm$ 70	100 $\pm$ 40 [*]	500 $\pm$ 60	600 $\pm$ 100	1400 $\pm$ 200 ^{***}
AMMT	18000 $\pm$ 4500	5700 $\pm$ 200 [*]	1000 $\pm$ 400 ^{***}	13000 $\pm$ 1600	8100 $\pm$ 1600	11000 $\pm$ 2800
AMMI	1300 $\pm$ 300	800 $\pm$ 200	60 $\pm$ 20 [*]	1100 $\pm$ 100	900 $\pm$ 300	1300 $\pm$ 300
MMAI	600 $\pm$ 200	500 $\pm$ 200	400 $\pm$ 100	600 $\pm$ 200	700 $\pm$ 300	3100 $\pm$ 300 ^{***}
DCPP	200 $\pm$ 30	200 $\pm$ 20	60 $\pm$ 10 ^{***}	200 $\pm$ 10	200 $\pm$ 10	300 $\pm$ 40 [*]
MM(R)NMA	1400 $\pm$ 400	900 $\pm$ 70	400 $\pm$ 50 ^{***}	400 $\pm$ 80	1400 $\pm$ 300	3200 $\pm$ 400 ^{***}
MM(S)NMA	700 $\pm$ 100	500 $\pm$ 100	300 $\pm$ 70	500 $\pm$ 200	800 $\pm$ 100	1300 $\pm$ 300 ^{***}
AEMMA	800 $\pm$ 100	700 $\pm$ 50	600 $\pm$ 60	600 $\pm$ 200	2800 $\pm$ 100 ^{***}	1700 $\pm$ 300
AEPCA	700 $\pm$ 10	400 $\pm$ 50	500 $\pm$ 100	400 $\pm$ 50	2500 $\pm$ 200 ^{***}	2600 $\pm$ 400 ^{***}
N-Dipropyl Indan	23000 $\pm$ 4400	9200 $\pm$ 600 ^{***}	1900 $\pm$ 300 ^{***}	18000 $\pm$ 1000	15000 $\pm$ 2000	32000 $\pm$ 2500

*
 $p < 0.05$ vs. hSERT

 $p < 0.001$ vs. hSERT.

Table 3

Cocaine analogue K_i values (nM) for [3 H]5-HT uptake inhibition at hSERT, and the TMH III mutants, I167L, A169D, F170I, I172M, S174M. [3 H]5-HT uptake assays were performed on transiently transfected HeLa cells, as described in Materials and Methods. Data represent the mean $\pm$ SEM for at least three independent experiments performed in triplicate, except RTI-311, n of 2.

	hSERT	I167L	A169D	F170I	I172M	S174M
cocaine	1000 $\pm$ 100	300 $\pm$ 60	300 $\pm$ 90	1300 $\pm$ 70	52000 $\pm$ 30000 *	800 $\pm$ 200
RTI-31	20 $\pm$ 4	20 $\pm$ 7	1.4 $\pm$ 0.1	30 $\pm$ 8	3300 $\pm$ 1500 ***	20 $\pm$ 1
RTI-32	120 $\pm$ 2	50 $\pm$ 10	7 $\pm$ 3	120 $\pm$ 20	13000 $\pm$ 3800 **	60 $\pm$ 20
RTI-55	1.3 $\pm$ 0.4	0.6 $\pm$ 0.2	1.1 $\pm$ 0.7	2.5 $\pm$ 0.3	170 $\pm$ 100	2.1 $\pm$ 0.4
RTI-83	40 $\pm$ 8	20 $\pm$ 4	7 $\pm$ 3	50 $\pm$ 7	5900 $\pm$ 1000 ***	20 $\pm$ 3
RTI-112	5 $\pm$ 0.6	2 $\pm$ 0.7	3 $\pm$ 1.0	7 $\pm$ 1.3	80 $\pm$ 10 ***	7 $\pm$ 0.6
RTI-121	100 $\pm$ 8	40 $\pm$ 2	10 $\pm$ 1	90 $\pm$ 7	2700 $\pm$ 1900	50 $\pm$ 20
RTI-147	6000 $\pm$ 1800	2800 $\pm$ 1000	500 $\pm$ 200	9700 $\pm$ 2100	132000 $\pm$ 17200 ***	7500 $\pm$ 900
RTI-311	20 $\pm$ 7	ND	ND	ND	8000 $\pm$ 200 *	ND
CPT	200 $\pm$ 60	ND	ND	400 $\pm$ 10	16000 $\pm$ 4000 ***	200 $\pm$ 40

* $p < 0.05$ vs. hSERT
 *** $p < 0.001$ vs. hSERT.

Table 4

Antidepressant K_i values (nM) for [3 H]5-HT uptake inhibition at hSERT, and the TMH III mutants, I167L, A169D, F170I, I172M, S174M. [3 H]5-HT uptake assays were performed on transiently transfected HeLa cells, as described in Materials and Methods. Data represent the mean $\pm$ SEM for at least three independent experiments performed in triplicate.

	hSERT	I167L	A169D	F170I	I172M	S174M
amitriptyline	10 $\pm$ 5	2 $\pm$ 1	20 $\pm$ 7	5 $\pm$ 3	400 $\pm$ 100 ^{***}	200 $\pm$ 70
amoxepine	300 $\pm$ 30	500 $\pm$ 200	800 $\pm$ 400	200 $\pm$ 20	1200 $\pm$ 100	400 $\pm$ 40
atomoxetine	200 $\pm$ 100	70 $\pm$ 9	50 $\pm$ 30	200 $\pm$ 100	5500 $\pm$ 2000 ^{***}	1100 $\pm$ 800
citalopram	10 $\pm$ 2	6 $\pm$ 3	30 $\pm$ 10	7 $\pm$ 2	4900 $\pm$ 2300 ^{***}	20 $\pm$ 3
clomipramine	0.3 $\pm$ 0.1	1.0 $\pm$ 0.4	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1	50 $\pm$ 20 ^{***}	3 $\pm$ 0.8
desipramine	100 $\pm$ 7	40 $\pm$ 10	20 $\pm$ 9	90 $\pm$ 10	900 $\pm$ 300 ^{***}	200 $\pm$ 50
doxepin	100 $\pm$ 40	500 $\pm$ 200	300 $\pm$ 200	200 $\pm$ 60	200 $\pm$ 20 ^{***}	300 $\pm$ 70
imipramine	5 $\pm$ 0.3	3 $\pm$ 0.3	5 $\pm$ 1	3 $\pm$ 1	400 $\pm$ 50 ^{***}	20 $\pm$ 1
fluoxetine	10 $\pm$ 3	10 $\pm$ 6	20 $\pm$ 7	20 $\pm$ 7	700 $\pm$ 200 ^{***}	20 $\pm$ 5
fluvoxamine	10 $\pm$ 4	8 $\pm$ 3	5 $\pm$ 1	10 $\pm$ 7	60 $\pm$ 20 ^{***}	90 $\pm$ 20
maprotiline	16000 $\pm$ 4000	8400 $\pm$ 2000	29000 $\pm$ 10000	15000 $\pm$ 4000	16000 $\pm$ 9000	20000 $\pm$ 8000
nisoxetine	200 $\pm$ 50	200 $\pm$ 60	70 $\pm$ 30	200 $\pm$ 100	23000 $\pm$ 11000 ^{***}	400 $\pm$ 100
nortriptyline	70 $\pm$ 7	40 $\pm$ 7	40 $\pm$ 20	50 $\pm$ 20	900 $\pm$ 200 ^{***}	300 $\pm$ 40
norclomipramine	20 $\pm$ 8	5 $\pm$ 1	20 $\pm$ 10	9 $\pm$ 1	600 $\pm$ 200 ^{***}	60 $\pm$ 30
protriptyline	90 $\pm$ 4	1800 $\pm$ 1000	200 $\pm$ 50	200 $\pm$ 80	2500 $\pm$ 700 [*]	290 $\pm$ 90
sertraline	1 $\pm$ 0.3	2 $\pm$ 1.0	0.8 $\pm$ 0.3	3 $\pm$ 1	80 $\pm$ 30 ^{***}	2 $\pm$ 0.2
trimipramine	400 $\pm$ 200	1600 $\pm$ 300	1800 $\pm$ 500	300 $\pm$ 100	6700 $\pm$ 1400 ^{***}	600 $\pm$ 200
venlafaxine	40 $\pm$ 10	20 $\pm$ 7	10 $\pm$ 4	40 $\pm$ 2	19000 $\pm$ 4000 ^{***}	50 $\pm$ 20
zimetidine	100 $\pm$ 70	500 $\pm$ 300	600 $\pm$ 200	200 $\pm$ 30	9800 $\pm$ 2200 ^{***}	400 $\pm$ 70
3-alpha-bis	5000 $\pm$ 2000	5000 $\pm$ 3000	10000 $\pm$ 3000	6000 $\pm$ 2000	21000 $\pm$ 9000	7000 $\pm$ 30

*
 $p < 0.05$ vs. hSERT

 $p < 0.001$ vs. hSERT.