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Interactions of Tryptamine Derivatives with Serotonin Transporter Species Variants Implicate Transmembrane Domain I in Substrate Recognition

ERIKA M. ADKINS, ERIC L. BARKER, and RANDY D. BLAKELY

Department of Pharmacology and Center for Molecular Neuroscience, Vanderbilt University Medical Center, Nashville, Tennessee (E.M.A., R.D.B.); and Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University School of Pharmacy, West Lafayette, Indiana (E.L.B.)

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ABSTRACT

The serotonin (5-hydroxytryptamine, 5-HT) transporter (SERT) is responsible for the inactivation of synaptic 5-HT and is also a target for multiple psychostimulants. Despite the critical role of SERT in 5-HT inactivation and psychostimulant response, many aspects of the transporter's recognition of ligands are poorly defined. We took advantage of sequence divergence of SERT species variants to identify structural determinants of substrate recognition. Tryptamine derivatives with substitutions at the 4 and 7 positions on the phenyl ring, the indole nitrogen, and the β position show up to 40-fold potency differences for inhibiting [^{3}H]5-HT transport in cells transfected with either human or *Drosophila melanogaster* SERT cDNAs. Species selectivities of these derivatives were largely recapitulated in antagonist binding. Human/*D. melanogaster* SERT chimera studies implicated the first two SERT transmembrane domains (TMDs) in the potency of the indole nitrogen-substituted compounds *N*-isopropyltryptamine (NIT), 5-methoxy-*N*-isopropyltryptamine (5-MNIT), and the 7-substituted compound 7-benzyloxytryptamine (7BT). Potency differences of analogs with substitutions at the 4 and β positions are influenced by sequences distal to this region. Within TMD I-II, species-scanning mutagenesis implicated a single residue (Y95 in human SERT, F90 in *D. melanogaster* SERT) in the recognition of NIT, 5-MNIT, and 7BT. Remarkably, this is the same site we established previously in species-specific recognition of the antagonists citalopram and mazindol. These findings support a critical role for TMD I residues in defining shared aspects of SERT substrate and antagonist recognition.

Serotonin (5-hydroxytryptamine, 5-HT) is an important neurotransmitter and regulatory molecule involved in a wide variety of physiologic functions and behaviors, including vasoconstriction, platelet aggregation, gastric motility, sleep, appetite, and mood (Fozard, 1989; Sanders-Bush and Mayer, 1996; Lucki, 1998). The synaptic actions of 5-HT are primarily terminated by ion-coupled (Na^+ and Cl^-) 5-HT transporters (SERTs), carriers also found in specialized peripheral tissues including platelets, pulmonary epithelia, and placenta (Ramamoorthy et al., 1993; Barker and Blakely, 1995; Brownstein and Hoffman, 1997). In addition to translocating its endogenous substrate 5-HT, SERTs also transport a number of tryptamine derivatives, psychoactive substances, and serotonergic neurotoxins, including methylenedioxymethamphetamine (MDMA; "ecstasy"), 5,7-dihydroxytryptamine, *p*-chloroamphetamine, and fenfluramine (Horn, 1973, 1978; Baumgarten et al., 1975; Rudnick and Wall, 1992). SERTs are potently antagonized by many tricyclic antidepressants, including imipramine and amitriptyline, and are targets of the widely prescribed serotonin selective reuptake inhibitors (e.g., fluoxetine, citalopram, and paroxetine) (Barker and Blakely, 1995; Tatsumi et al., 1997). SERTs are members of the Na^+/Cl^--dependent transporter gene family (Povlock and Amara, 1997; Nelson, 1998), which includes the closely related dopamine and norepinephrine transporters (DAT and NET, respectively). Initial hydropathy analyses (Blakely et al., 1991; Hoffman et al., 1991) indicate these carriers to exhibit a 12-transmembrane-spanning topology with intracellular NH_2 and COOH termini, a model supported by direct biochemical studies (Chen et al., 1998).

Because transmembrane domains of the SERT gene family display significant sequence conservation, a common structural framework is likely for substrate binding and translocation. In turn, sequence divergence must contribute trans-

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ABBREVIATIONS: HT, hydroxytryptamine; SERT, serotonin transporter; MDMA, methylenedioxymethamphetamine; DAT, dopamine transporter; NET, norepinephrine transporter; dSERT, *Drosophila melanogaster* serotonin transporter; hSERT, human serotonin transporter; HEK, human embryonic kidney; TMD, transmembrane domain; 7-MT, 7-methyltryptamine; 7-BT, 7-benzyloxytryptamine; NIT, *N*-isopropyltryptamine; 5-MNIT, 5-methoxy-*N*-isopropyltryptamine.

porter-specific ligand recognition. Study of sequence divergence among transporter subtypes with different substrate preferences (e.g., GAT1 versus SERT) may thus reveal key determinants of substrate selectivity and antagonist specificity. For example, biogenic amine transporters (SERT, DAT, NET) possess an Asp residue in TMD I (D98 in rat and human SERT), whereas GAT1 has a Gly at this position. We have recently argued that D98 coordinates 5-HT through an ion-pairing interaction with the substrate's protonated amine (Barker et al., 1999). Obviously, this interaction, although a critical determinant of potency, cannot explain substrate selectivity for 5-HT over other indolealkylamines or with catecholamines. The identification of other points of contact between transporter and substrate would constrain models, define interhelical packing, and help delineate how the permeation pathway is organized.

Recently, we have illustrated how comparative pharmacologic studies with species variants of the same transporter and cross-species chimeras can elucidate key structural determinants of antagonist recognition (Barker and Blakely, 1998). Thus, studies of rat and human SERT chimeras demonstrated that a single residue in TMD XII is responsible for species-selectivity of tricyclic antidepressants (Barker et al., 1994; Barker and Blakely, 1996). More recently, we capitalized on the greater sequence and functional divergence of human versus *Drosophila melanogaster* SERTs (dSERT) to pinpoint a recognition site in TMD I for two structurally distinct competitive antagonists, mazindol and citalopram (Barker et al., 1998). We reasoned that this same approach might provide insight into those regions and residues in SERT that are responsible for the recognition of substrates and elucidate whether these sites are distinct from residues used by SERT antagonists.

In the present report, we demonstrate that hSERT and dSERT exhibit differential recognition of substituted tryptamines in both transport and binding assays. Chimera and site-directed mutagenesis studies establish a role for hydroxylation of a single aromatic residue in TMD1 in SERTs (Y95 in hSERT, F90 in dSERT) in determining species-selectivity of 7-substituted and indole nitrogen-substituted tryptamines. The physical proximity of Y95 to D98 reaffirms an involvement of TMD I in formation of the 5-HT permeation pathway. Furthermore, identity of this site with the residue found to influence mazindol and citalopram recognition suggests that these and structurally related competitive antagonists inhibit transport by directly occluding a binding site for 5-HT.

Experimental Procedures

Materials. Dulbecco's modified Eagle's medium was obtained from Fisher Scientific (Pittsburgh, PA), fetal bovine serum from Hyclone (Logan, UT), and HeLa cells from American Type Culture Collection (Manassas, VA). Trypsin, glutamine, penicillin/streptomycin, OptiMEM, and Lipofectin were purchased from Life Technologies (Gaithersburg, MD). The QuikChange Mutagenesis kit was purchased from Stratagene (La Jolla, CA). Vaccinia virus T7 RNA polymerase (vVT7–3) was provided by Dr. Bernard Moss (National Institute of Allergy and Infectious Diseases, Bethesda, MD). [^{3}H]5-HT, [^{3}H]mazindol, and [^{3}H]citalopram were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Ecoscint H and Optiphase SuperMix were purchased from National Diagnostics (Atlanta, GA) and Wallac (Gaithersburg, MD), respectively. Paroxetine was a gift from SmithKline Beecham; *N*-isopropyltryptamine and 5-methoxy-*N*-isopropyltryptamine were gifts from Lilly Research Laboratories (Indianapolis, IN). 1-Methyltryptamine, 1-methylserotonin, RU 24969, 5-hydroxy-7-methoxytryptamine, 4-hydroxytryptamine, 7-hydroxytryptamine, α-methyltryptamine, serotonin *o*-sulfate, and 5,6,7-trihydroxytryptamine were provided by Research Biochemicals International (RBI, Natick, MA) and SRI International (Menlo Park, CA) as part of the Chemical Synthesis Program of the National Institute of Mental Health (contract N01-MH30003). 2-Methylserotonin was purchased from RBI. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

SERT Expression Systems. hSERT stably transfected in HEK-293 cells was produced as described previously (Qian et al., 1997); dSERT and hSERT Y95F were similarly stably expressed in HEK-293 cells. Briefly, dSERT cDNA in pBluescript KSII$^+$ (Blakely et al., 1991) was excised with *Xho*I/*Xba*I and subcloned into pRC/CMV3 (Invitrogen, San Diego, CA). Stably transfected cell lines were produced by introducing the dSERT pcDNA3 into HEK-293 cells using liposome-mediated transfer (Lipofectin; Life Technologies, Gaithersburg, MD), as described by the manufacturer. Clonal populations of cells were selected with 250 mg/l geneticin (G418), and individual clones were tested for SERT substrate and antagonist potency. The clone that displayed the highest 5-HT uptake (D3), demonstrated a pharmacologic profile similar to dSERT transiently expressed in HeLa cells (Blakely et al., 1991) and was used in the experiments reported. hSERT Y95F (Barker et al., 1998) was excised from pBluescript SKII$^-$ with *Not*I/*Age*I and subcloned into pcDNA3 and cells stably transfected as with dSERT. The hSERT Y95F cell line was characterized in [^{3}H]5-HT transport assays for substrate and antagonist potency and was found to display values similar to those determined for hSERT Y95F in transiently-transfected HeLa cells (data not shown).

SERT Chimera Construction and Mutagenesis. Human/*D. melanogaster* chimeras were generated by a restriction site-independent method as described previously (Moore and Blakely, 1994; Barker et al., 1998). The focus of our current studies, the chimera designated D136, contains *D. melanogaster* sequence through amino acid residue 136, corresponding to the end of putative TMD II, and human sequence for the remainder of the protein (see Table 3). Site-directed mutagenesis to switch divergent residues in hSERT to their corresponding residue in dSERT is described in Barker et al. (1998). These mutations in the first putative TMD are S91A and Y95F; in TMD II, these are L119V, T122C, I123L, M124C, A125L, and I130L. These mutations constitute all the differences between hSERT and dSERT in TMDs I and II and the intervening loop. Double mutants containing the Y95F mutation in TMD I and each of the mutations in TMD II were constructed using the TMD II point mutant constructs as a template and engineered in the Y95F background using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA). Mutant segments were isolated via a *Xba*I/*Nsi* I digest and subcloned into parental hSERT pBSKII- construct. In all mutations, restriction sites were also introduced as silent mutations to initially identify mutagenic DNAs. Subsequent sequencing (Center for Molecular Neuroscience Neurogenomics and Sequencing Core Facility) confirmed the mutation and a lack of unintended sequence modification.

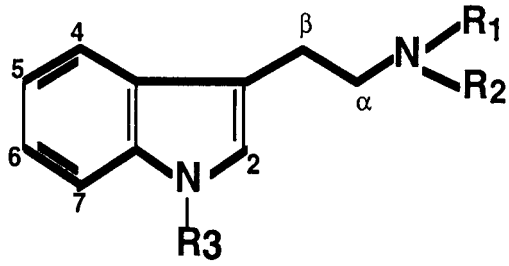
5-HT Transport Measurements. HeLa cells, maintained at 37°C in a 5% CO_2 humidified incubator and grown in HeLa complete media (Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin), were plated at a density of 100,000 cells per well in a 24 well culture plate. Cells were infected with a vaccinia virus carrying the T7 RNA polymerase in OptiMEM for 30 min at 37°C, after which SERT cDNAs cloned into pBluescript KS$^-$ (hSERT) or pBluescript SK$^+$ (dSERT) downstream of the T7 RNA polymerase promoter were transfected with 3 μl of lipofectin/mg of DNA, also in OptiMEM medium. After transfection (6 h), cells were assayed for [^{3}H]5-HT transport in Krebs/Ringers/HEPES assay buffer as described previ-

ously (Barker et al., 1999). Briefly, the cells were washed in assay buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM HEPES, pH 7.4), followed by a preincubation in 37°C assay buffer containing 1.8 g/l glucose. Cells were incubated for 10 min at 37°C with varying concentrations of unlabeled substrate derivative and 20 nM [^{3}H]5-HT, 100 mM pargyline, and 100 mM L-ascorbate. The reaction was terminated by washing 3 times with ice-cold assay buffer. The cells were dissolved in OptiPhase scintillation fluid and [^{3}H]5-HT accumulated determined by liquid scintillation counting on a Wallac Microbeta plate reader. Uptake in mock transfected cells was subtracted from SERT-transfected cells to determine specific uptake. IC_{50} values were derived using a nonlinear least-squares curve fit (Kaleidagraph, Synergy Software, Reading PA), and K_I values derived by application of Cheng-Prusoff assumptions (Cheng and Prusoff, 1973). Transport assays were performed in HEK-293 hSERT and dSERT stably transfected cells, as in transiently transfected HeLa cells, with the following changes. Stably transfected cells were maintained in complete medium containing 250 mg/l G418 sulfate and grown on poly-*d*-lysine coated 24-well plates. Specific binding was determined by subtracting data from cells treated with 50 μM paroxetine. All experiments were performed in duplicate or triplicate and repeated in three or more separate assays. Mean K_I values were logarithmically converted and analyzed using two-sided Student's *t* tests (GraphPad InStat for Macintosh, ver. 2.03; GraphPad, San Diego, CA), with $p < 0.05$ taken as significant.

Radioligand Binding. To determine whether K_I values estimated in uptake inhibition studies reflect ligand affinity or steps subsequent to ligand recognition in the transport cycle, we performed competition radioligand binding experiments with membranes prepared from hSERT and dSERT stably transfected HEK-293 cells. Cells were plated on 150-mm dishes and washed once with PBS, and then detached from the plate with a cell scraper in the same buffer. Cells were lysed using a Polytron (Brinkman Instruments, Westbury, NY) for 30 s at 18,000 rpm, and membranes were centrifuged for 30 min at 13,000 rpm. The resulting pellet was resuspended in binding buffer (50 mM Tris/100 mM NaCl, pH 8.0) containing protease inhibitors (250 μM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). The protein content of the membrane suspension was determined by the Bradford method (Bio-Rad, Hercules, CA). Previously (Barker et al., 1998), we have shown that citalopram (Schloss and Betz, 1995)

TABLE 1

Tryptamine derivative K_I values for hSERT and dSERT (micromolar). Derivatives of the parent compound tryptamine were examined for the ability to discriminate hSERT and dSERT in a [^{3}H] 5-HT competition uptake assay as described under *Experimental Procedures*. The tryptamine backbone is numbered to indicate the substitution position of the derivatives according to Horn (1978). K_I values were transformed from IC_{50} values by the Cheng-Prusoff equation (Cheng and Prusoff, 1973) and are the result of at least three independent experiments. Results are expressed as mean ± S.E.M.



Compound	Substitution	hSERT K_1	dSERT K_1
		μM	
Tryptamine		0.9 ± 0.1[a]	3.6 ± 0.5
4-Hydroxytryptamine	4	8.3 ± 1.4[a]	67.0 ± 6.6
5-Hydroxytryptamine	5	0.82 ± 0.09	1.1 ± 0.3
5-Methyltryptamine	5	4.9 ± 0.9[a]	17.9 ± 2.7
5-Methoxytryptamine	5	22.9 ± 6.2	15.8 ± 4.3
5-Carboxamidotryptamine	5	36.8 ± 8.1	22.4 ± 5.6
Serotonin o-sulfate	5	693.0 ± 108.0	236.0 ± 28.0
5-Hydroxytryptophol	5,amine	127.0 ± 20.3	282.0 ± 94.0
5-Methoxy-*N*,*N*-DMT	5,R1,R2	7.4 ± 0.8	8.6 ± 1.8
6-Fluorotryptamine	6	0.6 ± 0.2	1.2 ± 0.4
6-Methoxytryptamine	6	1.5 ± 0.2	1.1 ± 0.4
7-Hydroxytryptamine	7	6.8 ± 0.6	11.6 ± 2.0
7-Methyltryptamine	7	0.25 ± 0.03[a]	1.7 ± 0.2
7-Benzyloxytryptamine	7	1.3 ± 0.1[b]	0.31 ± 0.03
2-Methylserotonin	2,5	5.5 ± 0.2[a]	30.4 ± 5.1
5,6-Dihydroxytryptamine	5,6	27.8 ± 17.2	29.6 ± 6.5
5,7-Dihydroxytryptamine	5,7	11.6 ± 1.8	11.9 ± 1.4
5-Hydroxy-7-methoxytryptamine	5,7	6.8 ± 0.7[a]	24.9 ± 3.2
5,6,7-Trihydroxytryptamine	5,6,7	514.0 ± 140.0	866.0 ± 259.0
3-(β-Aminoethyl)-5-hydroxybenzothiophene	indole N	3.2 ± 0.5	4.0 ± 1.1
1-Methyltryptamine	R3	2.3 ± 0.3	3.5 ± 0.3
1-Methylserotonin	R3,5	2.8 ± 0.4[a]	6.0 ± 0.6
N-Isopropyltryptamine	R3	49.3 ± 4.3[b]	1.2 ± 0.2
5-Methoxy-*N*-isopropyltryptamine	R3,5	84.3 ± 6.5[b]	2.6 ± 0.6
N,*N*-Dimethyltryptamine	R1,R2	0.7 ± 0.1[a]	3.1 ± 0.4
α-Methyltryptamine	α	1.1 ± 0.0[b]	0.57 ± 0.05
5-Hydroxy-3-(1-methylpiperidin-4-yl)-1*H*-indole (BRL 54443)	β	2.6 ± 0.9	1.5 ± 0.3
5-Methoxy-3-(1,2,5,6-tetrahydro-4-pyridinyl)-1*H*-indole (RU 24969)	β	0.60 ± 0.09[a]	9.8 ± 1.4
3-(Dimethylaminomethyl)indole (Gramine)	β	25.3 ± 2.3	29.2 ± 2.7

[a] hSERT significantly greater than dSERT ($p < 0.05$).
[b] hSERT significantly less than dSERT ($p < 0.05$).

and mazindol (Javitch et al., 1984) are high-affinity, species-selective antagonists for human SERT and *D. melanogaster* SERT, respectively. Binding assays were performed with 50 μg of protein, 10 nM [^{3}H]mazindol (dSERT) or [^{3}H]citalopram (hSERT and Y95F), binding buffer, and varying concentrations of substrate derivative for 1 h at 25°C. Nonspecific binding for all constructs was determined in parallel incubations of membranes with 50 μM paroxetine. Nine-point competition isotherms were generated for each tryptamine derivative tested, assaying individual concentrations in duplicate. Membranes were collected on Brandel GF/B glass fiber filters, presoaked in 0.3% polyethylenimine, using a Brandel harvester. Filters were incubated overnight in Ecoscint H and emission from bound label determined on a liquid scintillation counter. Data were subjected to nonlinear least-squares curve fitting to a two parameter logistic equation for binding competition assuming a single population of SERT binding sites using GraphPad Prism. Mean K_I values were logarithmically converted and analyzed using two-sided Student's t tests, with $p < 0.05$ taken as significant.

Results

Human and *D. melanogaster* SERTs Differentially Discriminate Tryptamine Derivatives. SERT species variants, expressed in parallel in transiently transfected cells, exhibit different sensitivities to multiple transport inhibitors, properties that have allowed for the identification of potential antagonist contact sites (Barker et al., 1994, 1998; Barker and Blakely, 1996). In transport assays, unlabeled 5-HT was equipotent at inhibiting [^{3}H]5-HT uptake by hSERT and dSERT (Table 1), like many tryptamine analogs tested (<5-fold difference in K_I values). However, several tryptamine derivatives were identified that inhibited [^{3}H]5-HT uptake with significantly different potencies at hSERT and dSERT ranging from 5- to 40-fold (Table 1, Figs. 1 and 2). For example, the derivatives 4-hydroxytryptamine (8-fold), 7-methyltryptamine (7-MT; 7-fold), and RU 24969 (16-fold) demonstrated significantly greater potency at hSERT compared with dSERT ($p < 0.05$, Student's t test). Conversely, 7-benzyloxytryptamine (7-BT; 4-fold) and two indole nitrogen-substituted compounds, *N*-isopropyltryptamine (NIT; 40-fold) and 5-methoxy-*N*-isopropyltryptamine (5-MNIT; 32-fold) had significantly lower potency at hSERT compared with dSERT. All inhibition data obtained conformed to a single site model for 5-HT uptake inhibition (data not shown). In addition, we verified competitive inhibition kinetics for RU 24969 and NIT, finding that both compounds shifted the 5-HT transport K_m with no disruption in transport V_{max} (data not shown). These findings indicate that tryptamine analogs, including 5-HT, bind to a common site on SERTs and suggest that this site may be affected by the sequence divergence inherent in the two species variants.

Because the K_I values obtained from a transport assay contain determinants of both uptake and binding (Wood and Wyllie, 1982; Van Winkle, 1999), and we suspect that many of the analogs may be transported, we asked whether the potency differences observed among tryptamine derivatives relate to their intrinsic affinities using equilibrium binding assays. For these studies, we employed membranes from stably transfected HEK-293 cells in competition binding studies using the antagonists [^{3}H]mazindol (for dSERT) and [^{3}H]citalopram (for hSERT) with unlabeled tryptamines that demonstrated species differences in transport assays. Neither of these ligands, nor the alternatives we considered (e.g., ^{125}I-RTI-55 and [^{3}H]paroxetine), possess sufficient affinity with low nonspecific binding for the other species variant to allow for reliable measurement of displacement. In these assays, a small potency difference was evident for 5-HT (~3-fold) (Table 2). In contrast to what was initially observed in transport assays, 7-MT and 4-HT failed to discriminate between hSERT and dSERT in binding assays, suggesting that other determinants not preserved in membrane binding assays impact the potency of these compounds. However, as in uptake studies, significant potency differences were maintained in competition binding assays for RU 24969 (5-fold), 7-BT (6-fold), 5-MNIT (35-fold), and NIT (72-fold), and differences occurred with the same species preference as ob-

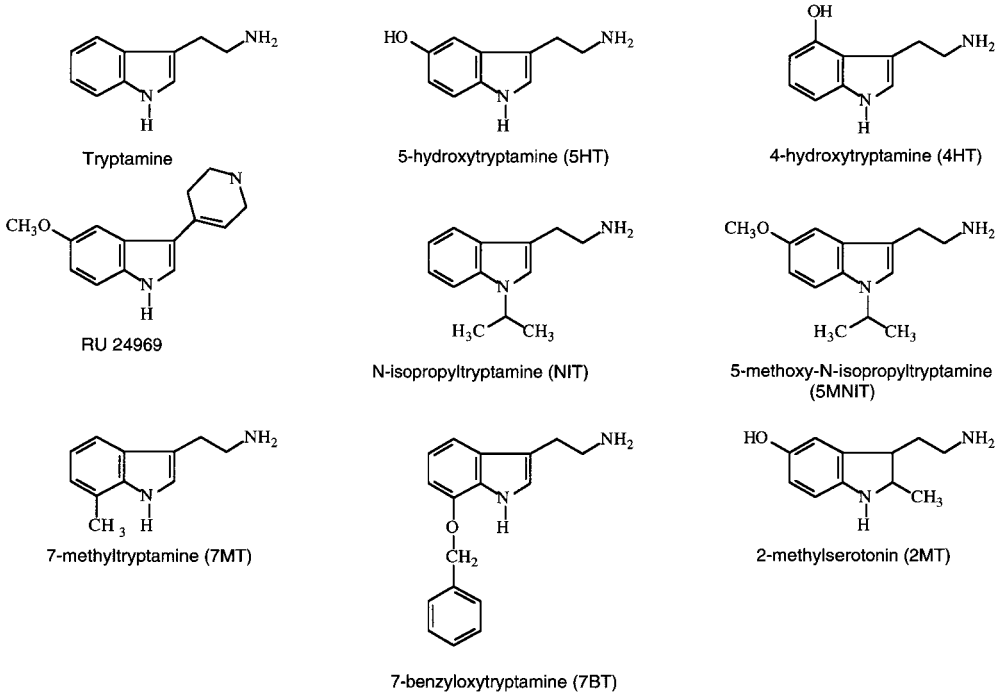


Fig. 1. Structures of selected tryptamine derivatives used in this study. All compounds except 5-HT displayed a 5-fold or greater difference in K_I values between hSERT and dSERT as assessed by transport assays in transiently transfected HeLa cells.

served in transport inhibition studies (Table 2). Although absolute potencies for inhibition of [^{3}H]5-HT uptake differed in comparing HeLa (Table 1) and HEK-293 (Table 2) transfected cells, relative differences between hSERT and dSERT were maintained. For RU 24969, 7-BT, 5-MNIT, and NIT, differences in ligand recognition are thus likely to contribute species-specific potency differences observed with uptake inhibition.

A Role for TMD I in SERT Interactions with Tryptamine Derivative Discrimination. Next we determined whether we could identify discrete regions of SERTs supporting the differential recognition of tryptamine derivatives. In particular, we ascertained the potency of 7-BT, NIT, 5-MNIT, and RU 24969 for [^{3}H]5-HT uptake inhibition using a human/*D. melanogaster* SERT chimera (Barker et al., 1998) expressed transiently in HeLa cells, and monitored in parallel with the two species variants. We have previously described chimera D136, which contains *D. melanogaster* sequence through the first two TMDs and human sequence for the remainder of the protein and expresses at or near the level of parental cDNAs (Barker et al., 1998). When tested with the compounds that display greater dSERT potency (NIT, 5-MNIT, and 7-BT), D136 showed a potency shifted toward the dSERT values (Table 3) even though the chimera possesses limited *D. melanogaster* sequence. This gain of function exhibited by NIT, 5-MNIT, and 7-BT at D136 relative to hSERT does not seem to arise from a nonspecific shift in potency as RU 24969, 4-HT, and 7-MT retained human-like potencies.

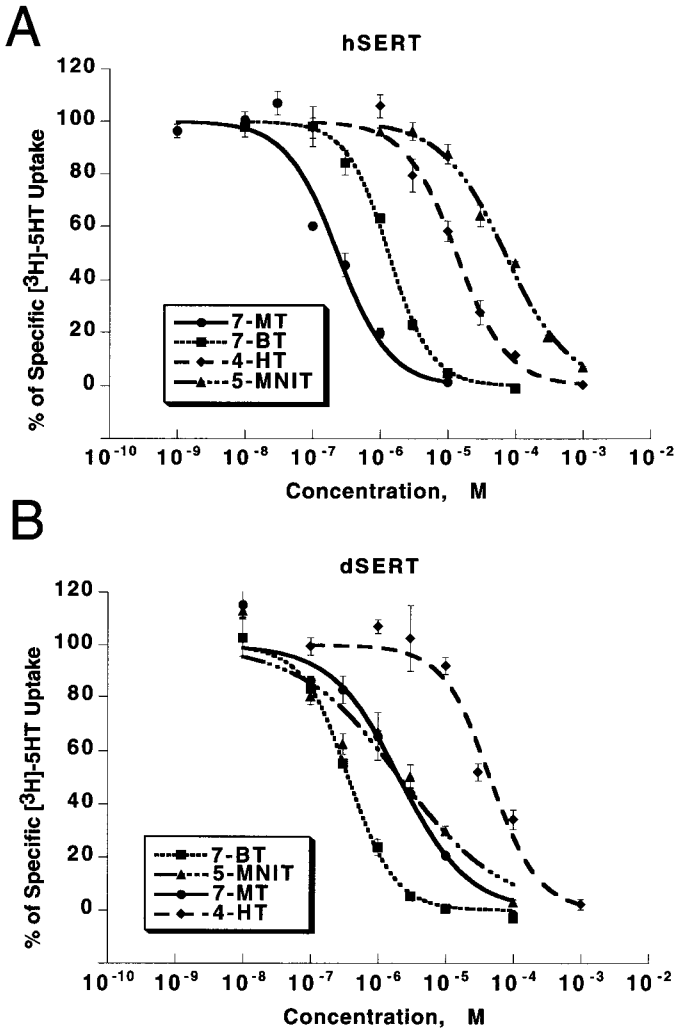


Fig. 2. Rank order potency for selected tryptamine derivatives at hSERT and dSERT. [^{3}H]5-HT transport assays were performed in transiently transfected HeLa cells as described under *Experimental Procedures*. Nonspecific uptake was determined from mock transfected cells and subtracted as background from the SERT-transfected cells. Total uptake was defined from SERT transfected cells that contained no competitive substrate. Data from representative experiments were plotted as the means of the percentage of specific 5-HT uptake ± S.E. of triplicate determinations from representative experiments. A, evaluation of 7-MT, 7-BT, 4-HT, and 5-MN potency for hSERT. The rank order potency for hSERT is 7-MT > 7-BT > 4-HT > 5-MN (K_I values were 0.23 ± 0.05 μM, 1.33 ± 0.08 μM, 13.1 ± 0.08 μM, and 69.2 ± 6.4 μM, respectively). B, evaluation of 7-MT, 7-BT, 4-HT, and 5-MN potency for dSERT. The rank order potency for dSERT is 7-BT > 5-MN = 7-MT > 4-HT (K_I values were 0.36 ± 0.02 μM, 2.1 ± 0.9 μM, 2.1 ± 0.5 μM, and 44.8 ± 8.3 μM, respectively).

The limited sequence difference between D136 and hSERT allowed us to investigate specific amino acids of D136 that contribute to the gain of potency exhibited by NIT, 5-MNIT, and 7-BT. We reasoned that critical differences in ligand interactions were likely to occur at the level of TMD I-II rather than the cytoplasmic NH_2 terminus, because tail swap chimeras between SERT and NET did not impact antagonist recognition (Blakely et al., 1993), and we had previously implicated a TMD I residue in recognition of the alkylamine chain of serotonin derivatives (Barker et al., 1999). Thus, mutant hSERT cDNAs carrying point mutations that result in switches of individual residues to a *D. melanogaster* identity in the TMD I-II region were examined for tryptamine derivative sensitivity in transport inhibition assays. hSERT S91A, L119V, T122C, I123L, M124F, A125L, and I130L mutations resulted in no shift in potency toward that observed for dSERT for either NIT, 5-MNIT, or 7BT. However, Y95F, an interconversion in TMD I, recapitulated and even exceeded the gain of potency seen with the chimera (Table 3, Fig. 3) for both the *N*-isopropyl compounds and 7-BT. Importantly, RU 24969, 7-MT, and 4-HT were unaffected by the Y95F mutation (Table 3), nor were these potencies influenced by any other mutation made in this region (data not shown).

Although the Y95F mutation mirrored the shift in K_I values observed for both *N*-isopropyl compounds in the D136 chimera, the shift for 7-BT seen with Y95F was actually greater than expected from D136 chimera studies. We hypothesized that 7-BT might be recruiting an additional contact in hSERT Y95F in TMD I-II of hSERT lacking in D136. We also noticed that one switch in this region, hSERT A125L, significantly reduced potency of 7-BT (Table 4), rather than increasing potency. Moreover, when the Y95F/A125L double mutation was analyzed, a potency similar to D136 was obtained. Double mutants between Y95F and other divergent residues lacked the restoration of D136 potency. These data suggests that the potency of 7-BT in D136 is influenced by contributions from both TMD I and TMD II.

Finally, to ascertain whether the behavior of hSERT Y95F with NIT and 7-BT was similar in both binding and transport, we used Y95F stably transfected HEK-293 cells in competition binding assays as described above. Although the increase was not as great as that observed in transport assays, a gain in binding potency also was evident with Y95F for NIT (Table 3, Fig. 4, binding K_I values: hSERT, 43.0 ± 8.0 μM; dSERT, 0.6 ± 0.0 μM; Y95F, 17.0 ± 1.0 μM). However, the increase in potency of 7BT for Y95F almost reproduced

TABLE 2

Comparison of K_I values of selected tryptamine derivatives for hSERT and dSERT in competition binding vs. transport assays. K_I values for membrane binding in hSERT and dSERT HEK-293 cells were determined from uptake inhibition and competition radioligand binding studies as described under *Experimental Procedures*, and are the result of at least three independent experiments. [^{3}H]5-HT uptake assays were performed in HEK-293 hSERT and dSERT stably transfected cells using methods similar to those described previously for transiently transfected HeLa cells. Results are presented as mean ± S.E.M.

Compound	K_I			
	HEK hSERT Binding	HEK dSERT Binding	HEK hSERT Uptake	HEK dSERT Uptake
		μM		
5-Hydroxytryptamine	4.8 ± 0.5	17.4 ± 2.2[a]	5.1 ± 0.7	5.4 ± 0.8
4-Hydroxytryptamine	118.3 ± 56.8	73.6 ± 44.3	46.0 ± 7.1	186.0 ± 29.0[b]
7-Methyltryptamine	2.40 ± 0.06	5.1 ± 1.3	2.9 ± 0.6	14.0 ± 2.6[b]
7-Benzyloxytryptamine	6.8 ± 1.5	1.1 ± 0.2[a]	4.3 ± 0.5	1.0 ± 0.3[b]
N-Isopropyltryptamine	43.0 ± 8.0	0.6 ± 0.0[a]	123.0 ± 26.0	4.1 ± 0.7[b]
5-Methoxy-*N*-isopropyltryptamine	84.8 ± 5.8	2.4 ± 0.8[a]	250.0 ± 55.0	8.9 ± 1.3[b]
5-Methoxy-3-(1,2,5,6-tetrahydro-4-pyridinyl)-1*H*-indole (RU 24969)	1.20 ± 0.05	5.9 ± 0.5[a]	5.4 ± 0.7	38.9 ± 2.4[b]

[a] $p < 0.05$, compared with hSERT value for binding.
[b] $p < 0.05$, compared with hSERT value for uptake.

the gain of function demonstrated in transport assays. (Table 3, Fig. 4, binding K_I values: hSERT, 6.8 ± 1.5 μM; dSERT, 1.1 ± 0.2 μM; Y95F, 1.3 ± 0.2 μM).

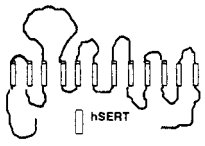
Discussion

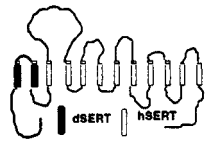
Whereas several studies have explored candidate regions (Barker et al., 1994; Buck and Amara, 1994; Giros et al., 1994; Lee et al., 1998; Vaughan et al., 1999) and residues (Kitayama et al., 1993; Baldessarini, 1996; Lin et al., 1999; Itokawa et al., 2000) dictating antagonist recognition by neurotransmitter transporters, the structure defining the substrate binding pocket is not well understood. Studies using DAT/NET chimeras point to the middle region of the transporter, TMDs IV-IX, containing structural determinants for substrate translocation (Buck and Amara, 1994; Giros et al., 1994). Data from cysteine-scanning studies in the third TMD of rat SERT suggest that an Ile residue is involved in 5-HT recognition (Chen et al., 1997). Also in TMD III, a Tyr residue has been implicated in substrate interactions in SERT, GAT1, and GLYT2 (Bismuth et al., 1997; Chen et al., 1997; Ponce et al., 2000). Species-scanning mutagenesis in DAT implicates a Val residue in the homologous position for DA and MPP+ interaction (Lee et al., 2000). NET and DAT proteins are sensitive to mutations at an Asp residue in the TMD I (Kitayama et al., 1992; Barker et al., 1999), which is absolutely conserved among the monoamine transporters. For the most part, these studies rely on the manipulation of protein determinants to suggest potential substrate interaction sites. Additional evidence for direct interactions can be gathered through the complementary use of modified ligands. For example, evidence from our lab supports a role for the TMD I conserved Asp in ion pairing with the protonated amine on 5-HT, a result obtained using site-directed mutagenesis in concert with a tryptamine derivative that contains a shortened alkylamine side chain, gramine (Barker et al., 1999).

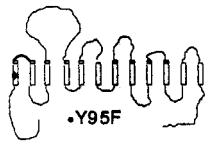
We used species variants of SERT to search for structural differences in substrate recognition and structural variants in the endogenous substrate tryptamine to potentiate the power of this experimental system (Fig. 1). Earlier results from our lab indicate that although SERT is unable to func-

TABLE 3

Comparison of tryptamine derivatives K_I values for hSERT, dSERT, the D136 chimera, and the hSERT Y95F point mutant. Chimera and point mutant construction are detailed under *Experimental Procedures*. K_I values were determined from competition uptake assays in transiently transfected HeLa cells as described under *Experimental Procedures*. K_I values were transformed from IC_{50} values by the Cheng-Prusoff equation (Cheng and Prusoff, 1973) and are the result of at least three independent experiments. Results are presented as mean ± S.E.M.







Compound	hSERT K_I	dSERT K_I	Chimera D136 K_I	hSERT Y95F K_I
		μM		
5-Hydroxytryptamine	0.82 ± 0.09	1.1 ± 0.3	0.51 ± 0.05[a]	0.4 ± 0.1[a,b]
4-Hydroxytryptamine	8.3 ± 1.4	67.0 ± 6.3[a]	11.5 ± 0.1[b]	5.5 ± 1.1[b]
7-Methyltryptamine	0.25 ± 0.03	1.7 ± 0.2[a]	0.11 ± 0.01[a,b]	0.17 ± 0.03[b]
7-Benzyloxytryptamine	1.3 ± 0.1	0.31 ± 0.03[a]	0.41 ± 0.03[a]	0.17 ± 0.01[a,b]
N-Isopropyltryptamine	49.3 ± 4.3	1.2 ± 0.2[a]	7.0 ± 0.6[a,b]	10.0 ± 1.9[a,b]
5-Methoxy-*N*-isopropyltryptamine	84.3 ± 6.5	2.6 ± 0.6[a]	10.3 ± 1.3[a,b]	6.1 ± 1.6[a,b]
5-Methoxy-3-(1,2,5,6-tetrahydro-4-pyridinyl)-1H-indole (RU 24969)	0.60 ± 0.09	9.8 ± 1.4[a]	0.6 ± 0.2[b]	0.7 ± 0.1[b]

[a] $p < 0.05$, compared with hSERT value.
[b] $p < 0.05$, compared with dSERT value.

tion as a chimera with other monoamine transporters, interspecies SERT chimeras are functional and can provide valuable information regarding antagonist interaction sites (Barker et al., 1994, 1998; Barker and Blakely, 1996). Not only did these studies locate potential interaction sites for tricyclic antidepressants (TMD XII) and heterocyclic antagonists (TMD I) but also provided evidence that ligand analogs can aid in more rigorously defining direct ligand-protein interactions. Thus we were able to associate the presence or absence of a ring hydroxyl on mazindol/mazindane with discrimination of the hydroxylated aromatic ring at hSERT Y95F A similar method has also been employed previously with rat and human 5-HT_{2A} receptors and N1-substituted ergolines and tryptamines, identifying likely determinants of the serotonin binding pocket (Johnson et al., 1994). In principle, an iterative application of this strategy can reveal spatial constraints for transporter residues located on nonadjacent TMDs making up the SERT permeation pathway.

We observed that some, but not all, substituted tryptamines display potency differences comparing human and *D. melanogaster* transporters, resulting in hSERT- and dSERT-specific rank-order potency profiles (Fig. 1). For example, we investigated many different types of substitutions at the 5 position, including halogenated derivatives, sulfate groups, methoxy groups, and carboxamido groups; although all had varying affects on the absolute potencies for hSERT or dSERT compared with the potency of tryptamine, the affinity shifts were in the same direction and of similar magnitude (Table 1). These shifts may indicate that the orientation of the 5 position of the 5-HT molecule to SERT results in the substrate encountering the same or similar amino acids in both hSERT and dSERT. Conserved residues may also interact with the 6 position and the amine nitrogen. Conservation of amino acids oriented toward the 5 position and the amine group would not be surprising, as it has long been established that these two positions are highly critical for the potency of serotonin for the transporter (Horn, 1973, 1978; Baumgarten et al., 1975; Chang et al., 1993).

Even considering the tryptamine variants we found to shift species selectivity, not all substitutions at these positions result in a differentiation between species (Table 1). This phenomenon may be explained for some derivatives by examining the size of substituted functional group. Thus, whereas 7-BT displays a 5-fold potency difference between human and *D. melanogaster* transporters, 7-hydroxytryptamine shows little or no difference in K_I values for the two species. Similarly, 1-methyltryptamine and 1-methylserotonin are substituted at the indole nitrogen, as are the com-

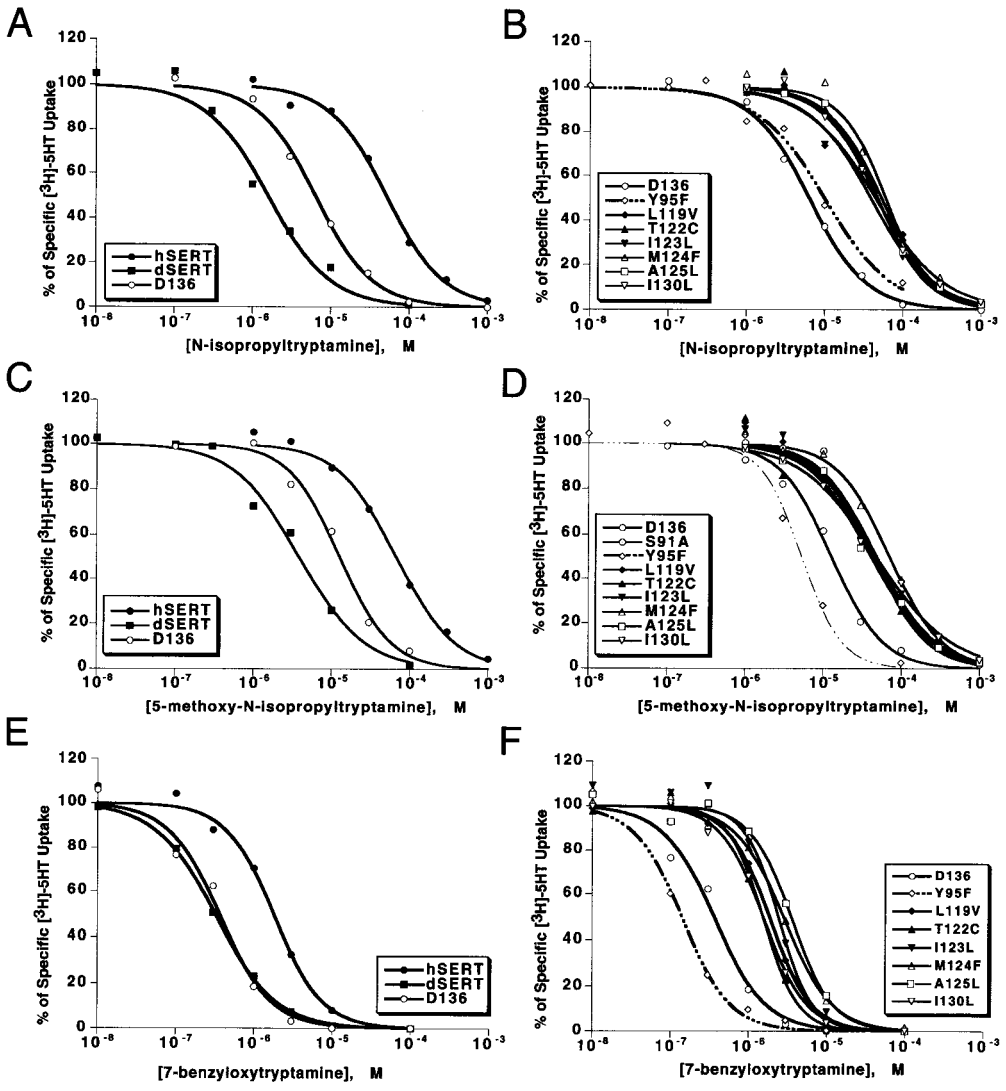


Fig. 3. Investigation of a substrate interaction site on SERT using a human/*D. melanogaster* chimera and site-directed mutagenesis. [^{3}H]5-HT transport assays were performed in transiently transfected HeLa cells as described under *Experimental Procedures*. Nonspecific uptake was determined from mock transfected cells and subtracted as background from the SERT transfected cells. Total uptake was defined from SERT-transfected cells that contained no competitive substrate. Data were plotted as the means of the percentage of specific 5-HT uptake ± S.E. of triplicate determinations from a representative experiment. The K_I values are reported in micromolar. A, inhibition of hSERT, dSERT, and D136 [^{3}H]5-HT transport by NIT. hSERT, 51.1 ± 4.0; dSERT, 1.6 ± 0.3; D136, 6.3 ± 0.5. B, inhibition of Y95F, L119V, T122C, I123L, M124F, A125L, and I130L [^{3}H]5-HT transport by NIT. Y95F, 10.0 ± 1.5; L119V, 44.3 ± 6.2; T122C, 53.0 ± 5.9; I123L, 38.5 ± 4.4; M124F, 60.0 ± 7.6; A125L, 47.4 ± 2.4; I130L, 46.1 ± 3.4. C, inhibition of hSERT, dSERT, and D136 [^{3}H]5-HT transport by 5-MNIT. hSERT, 66.8 ± 5.4; dSERT, 3.8 ± 0.5; D136, 12.6 ± 1.1. D, inhibition of S91A, Y95F, L119V, T122C, I123L, M124F, A125L, and I130L [^{3}H]5-HT transport by 5-MNIT. S91A, 70.0 ± 5.2; Y95F, 5.3 ± 0.7; L119V, 43.9 ± 6.7; T122C, 47.9 ± 6.3; I123L, 43.6 ± 8.0; M124F, 46.8 ± 3.6; A125L, 46.8 ± 6.5; I130L, 40.4 ± 3.4. E, inhibition of hSERT, dSERT, and D136 [^{3}H]5-HT transport by 7-BT. hSERT, 1.8 ± 0.2; dSERT, 0.32 ± 0.01; D136, 0.36 ± 0.05. F, inhibition of Y95F, L119V, T122C, I123L, M124F, A125L, and I130L [^{3}H]5-HT transport by 7-BT. Y95F, 0.14 ± 0.02; L119V, 1.9 ± 0.06; T122C, 1.5 ± 0.1; I123L, 2.4 ±0.3; M124F, 2.8 ± 0.2; A125L, 3.5 ± 0.3; I130L, 1.5 ± 0.1.

pounds NIT and 5-MNIT, but the methylated derivatives have approximately the same K_I values for both dSERT and hSERT, whereas the bulkier isopropyl derivatives have affinity differences of 40-fold for the two species. These potency differences suggest that the indole nitrogen faces different residues in the permeation pathway of hSERT and dSERT that are only detected with large, bulky substitutions. Importantly, a methoxy group at the 5 position does not result in significantly different potencies for hSERT and dSERT; this lends support to the idea that it is the isopropyl substitution on the indole nitrogen alone that leads to the large differences in potency for 5-MNIT for hSERT and dSERT. Finally, it is noteworthy that those compounds that demonstrated species-selectivity were not simply more potent for hSERT or dSERT, but exhibited mixed behavior. RU 24969, 4 MT, and 7-MT were more potent for hSERT than dSERT, whereas NIT, 5-MNIT, and 7-BT were more potent for dSERT than hSERT. This behavior suggests that it is the interaction of tryptamine substituents with divergent determinants of the binding pocket that determines ligand potency rather than general differences in conformational organization of the two carriers.

Previous success in our lab using interspecies chimeras of SERT to identify regions of ligand recognition (Barker et al., 1994, 1998; Barker and Blakely, 1996) led us to employ this method using hSERT/dSERT chimeras. Results from transport assays with the D136 chimera, containing *D. melanogaster* sequence from the N terminal through the second TMD, and the remainder of the protein human sequence, displayed a shift in potency toward the dSERT K_I value for 7-BT and the indole nitrogen substituted derivatives NIT and 5-MNIT, with no shift for 7-MT, 4-HT, or RU 24969 (Table 3, Fig. 3). Our results identified hSERT Y95F as responsible for the majority of the species specificity evident for all three compounds in the D136 chimera (Table 3, Fig. 3). Remarkably, this residue was previously identified to be largely responsible for the differential recognition by hSERT and dSERT of the antagonists mazindol and citalopram (Barker et al., 1998). In this latter study, we deduced that the hydroxyl group on hSERT Y95 led to steric interference with the mazindol hydroxyl group, thus accounting for mazindol's greater potency for dSERT. As the benzyloxy and isopropyl moieties of 7BT and NIT/5-MNIT, respectively, are on the same face of the molecule, and are freely rotatable, bulky groups, we suspect that both compounds interact with this aromatic residue in the first TMD. 7-BT, NIT, and 5-MNIT demonstrate a greater potency for dSERT (containing a Phe at position 90) than hSERT (containing a Tyr at position 95), Perhaps the hydroxyl group on the Tyr participates in direct steric interference with the substituted tryptamines. Another possibility is that the aromatic ring of dSERT F90 is oriented differently than hSERT Y95, probably through interior intrahelix hydrogen bonding, removing a negative interaction in the dSERT permeation pathway. The observa-

TABLE 4

Effect of hSERT/dSERT TMD II divergent residues on 7-BT species selectivity. [^{3}H]5-HT transport assays were performed in hSERT, dSERT, D136, Y95F, or double mutant transiently transfected HeLa cells. The Y95F/TMD II double mutants were constructed as detailed under *Experimental Procedures*. K_I values were determined from competition uptake assays in transiently transfected HeLa cells and are the results of at least three independent experiments. Results are presented as mean ± S.E.M.

Construct	7-BT K_I
	μM
hSERT	1.3 ± 0.1
dSERT	0.32 ± 0.03
D136	0.41 ± 0.03
hSERT Y95F	0.17 ± 0.01
L119V	1.9 ± 0.6
Y95F/L119V	0.22 ± 0.03
T122C	1.5 ± 0.2
Y95F/T122C	0.13 ± 0.01
I123L	2.2 ± 0.5
Y95F/I123L	0.14 ± 0.07
M124F	2.0 ± 0.8
Y95F/M124F	0.08 ± 0.01
A125L	3.6 ± 0.1[a]
Y95F/A125L	0.28 ± 0.01[b]
I130L	1.1 ± 0.2
Y95F/I130L	0.11 ± 0.01

[a] Significantly greater than hSERT ($p < 0.001$).
[b] Significantly greater than Y95F ($p < 0.001$).

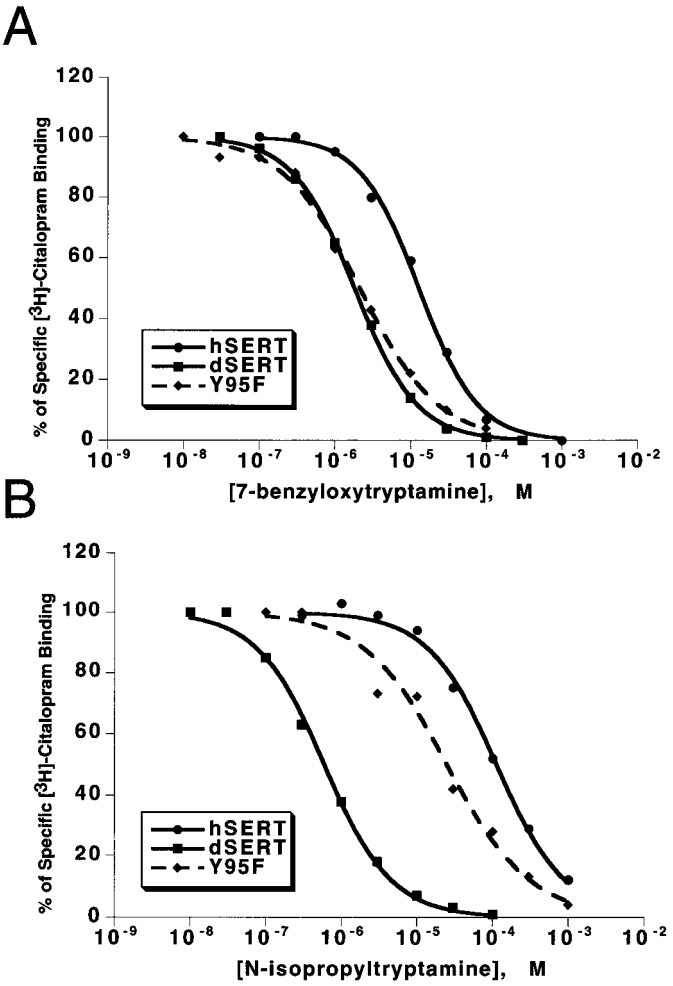


Fig. 4. Binding characterization of hSERT Y95F with 7-BT and NIT. Competition binding assays were performed on membranes of hSERT Y95F stably transfected HEK-293 cells as described under *Experimental Procedures*. Cell membranes were incubated with [^{3}H]citalopram for 60 min in the presence of increasing concentrations of 7-BT or NIT as a competitor. Nonspecific binding was defined with 50 μM paroxetine. Binding isotherms were fitted using nonlinear regression and K_I values derived from Cheng-Prusoff assumptions. Results are from representative experiments. A, 7-BT shifts Y95F to the dSERT-like value. K_I values: hSERT, 12.8 ± 0.7 μM; dSERT, 1.82 ± 0.04 μM; Y95F, 2.2 ± 0.2 μM. B, NIT shifts Y95F toward the dSERT-like value. hSERT, 112 ± 8 μM; dSERT, 0.60 ± 0.04 μM; Y95F, 24.7 ± 4.7 μM.

tion of lower potency between hSERT and dSERT for compounds such as mazindol, NIT, 5-MNIT, and 7BT, but not for the smaller 1-methyltryptamine or 7-hydroxytryptamine, could thus be explained by steric interference in hSERT. The role of Y95 and other TMD I residues as contributing to a substrate permeation pathway is now being investigated using the substituted cysteine accessibility method (Adkins and Blakely, 1999).

Binding studies with hSERT Y95F with 7-BT and NIT recapitulated the findings of transport assays (Fig. 4). Interestingly, NIT binding at hSERT Y95F shows a smaller shift toward the dSERT-like value than in transport assays. It is conceivable that this smaller effect of the Y95F mutation is caused by the aforementioned difference in conformational states occupied by SERT in membrane preparations, whereas in transport assays SERT is freely moving, or constrained to one conformation by ion gradients and membrane potential. In this regard, differential exposure of introduced cysteines in DAT has been recently noted by Chen et al. (2000) in comparison of binding versus transport assays. It is also possible that differences between NIT and 7-BT reflect differential activity of these tryptamine derivatives as substrates. Further studies using efflux assays or substrate-activated currents may help clarify this issue.

The presence of dSERT sequence in chimera D136 did not shift affinity for compounds with species-selective substitutions at the β and 4 positions (RU 24969 and 4-HT), in contrast to those compounds substituted at the indole nitrogen and the 7 position (Table 3). Because the chimera contains hSERT sequence distal to TMD II, a reasonable conclusion can be drawn that these particular substitutions are interacting with a region of SERT outside TMDs I and II. Also, regions distal to TMDs I-II are likely to contribute to the differential potency of NIT and 5-MNIT not accounted for by hSERT Y95F. Location of these distal sites should set physical constraints on interhelical packing, in that they should be limited by the 8-to 20-Å size of the tryptamine compounds used.

Although it may be premature to suggest a model of 5-HT interaction with TMD I residues, our data are consistent with the positioning of particular TMD I residues near specific functional groups of 5-HT. Previous results suggest the ion pairing of the conserved Asp residue with the amine nitrogen (Barker et al., 1999). The aromatic residue that we have found to be a critical determinant of both substrate and antagonist potency is one putative helix turn away from this Asp. It is possible to orient the amine nitrogen toward the Asp side chain, and the indole nitrogen facing Y95 (hSERT)/F90 (dSERT). Steric interference can therefore arise from the interactions of a bulky isopropyl group on the indole nitrogen and a Tyr hydroxyl group that could not occur with a Phe. This could lead to the decreased potency of hSERT relative to dSERT for the *N*-isopropyl derivatives. Precedent for this model comes from previous evidence that suggests a steric interaction of Y95, but not F90, with a functional group (hydroxyl) on mazindol (Barker et al., 1998). This model also orients the 4 and β positions of the tryptamine away from TMD I, consistent with species-selectivity of substituted tryptamines at these positions being defined by more distal helices. Recent reports suggest the involvements of TMDs III, XI, and XII in ligand recognition (Barker and Blakely, 1996; Chen et al., 1997; Mitsuhata et al., 1998; Lee et al., 2000), and it may be expected that the use of the combined method of species variants and ligand analogs may further define contributions to the serotonin binding pocket.

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Send reprint requests to: Randy D. Blakely, Ph.D., Rm 419, MRBII, Center for Molecular Neuroscience, Vanderbilt University Medical Center, Nashville, TN 37232-6420. E-mail: randy.blakely@mcmail.vanderbilt.edu