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Identification of a Single Amino Acid, Phenylalanine 586, That Is Responsible for High Affinity Interactions of Tricyclic Antidepressants with the Human Serotonin Transporter

ERIC L. BARKER and RANDY D. BLAKELY

Department of Pharmacology and Center for Molecular Neuroscience, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-6600

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SUMMARY

When assayed in parallel using transfected mammalian cells, human and rat serotonin transporters (SERTs) exhibit consistent differences in potency for tricyclic antidepressants but not for 5-hydroxytryptamine, cocaine, or nontricyclic serotonin transporter-selective reuptake inhibitors. Previously, using chimeric proteins, we determined that domains or residues distal to transmembrane domain 11 (amino acid 531) dictate the increased sensitivity of human SERT to imipramine. Using an additional chimera and site-directed mutagenesis, we have determined that a single amino acid, F586, is responsible for increased sensitivity to imipramine, desipramine, and nortriptyline. Thus, mutation of wild-type rat SERT (V586) to the human SERT identity F586, but no other divergent amino acids between human and rat SERTs, selectively increased tricyclic antidepressant potency. A reciprocal reduction in potency was observed when human SERT F586 was converted to the cognate rat SERT residue (V586). Interactions with other SERT antagonists, including paroxetine and cocaine, as well as the SERT substrates 5-hydroxytryptamine and *d*-amphetamine were unaffected by interconversion of this residue. Phenylalanine conversion in the human norepinephrine transporter at the homologous position failed to alter tricyclic inhibition of catecholamine uptake, revealing a SERT-specific context for use of the aromatic side chain at this position. Additional constraints on aromaticity at rat SERT position 586 were revealed by conversion of rat SERT V586 to Y586, which failed to replicate the effect of the F586 mutation. In addition, conversion to V586D, but not V586R, increased tricyclic potency to that of human SERT and additionally increased potency for cocaine but not paroxetine. These results implicate distal domains and a single residue in TMD 12 in the formation of high affinity SERT antagonist binding sites.

Transport of the neurotransmitter 5-HT back into presynaptic neurons regulates the concentration of synaptic 5-HT after release. This uptake process is mediated by Na^+- and Cl^--dependent SERTs, which are the molecular targets for many clinically effective antidepressants as well as drugs of abuse, including amphetamines and cocaine (1). Despite an emphasis on identifying SERT-selective reuptake inhibitors for the treatment of affective disorders, relatively little is known about the molecular determinants of the protein involved in ligand binding. Extensive biochemical and pharmacological studies in both brain and isolated tissue preparations have generated data demonstrating that most antagonists are competitive inhibitors of 5-HT uptake and possess mutually exclusive, overlapping, binding domains on SERTs (2, 3). For example, Graham *et al.* (2) used protection against the alkylating agent *N*-ethylmaleimide to argue for common and/or overlapping binding sites for both tricyclic and nontricyclic SERT antagonists at the 5-HT recognition site. However, kinetic analysis of radioligand dissociation rates using native preparations as well as heterologously expressed SERT cDNAs suggests heterogeneity in the binding of SERT antagonists, which is supportive of distinct ligand-specific contact sites (3, 4). Confirmation of tricyclic, nontricyclic, and cocaine binding sites on the SERT protein itself has been provided by molecular cloning and heterologous expression of SERT cDNAs in mammalian cells, where pharmacological sensitivities essentially equivalent to those of native SERT-containing tissues have been observed (5–7). SERTs are members of the γ-aminobutyric acid transporter/NET gene family of Na^+/Cl^--dependent transport proteins, which are characterized by 12 putative TMDs with intracellular amino and carboxyl termini. Mutation and heterologous expression of SERT cDNAs allow for determination of SERT domains responsible for substrate and antagonist recogni-

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); SERT, serotonin transporter; NET, norepinephrine transporter; DAT, dopamine transporter; DA, dopamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KRH buffer, Krebs-Ringers-HEPES buffer; TMD, transmembrane domain; RTI-55, 3β-(4-iodophenyl)tropan-2β-carboxylic acid methyl ester.

tion, to permit more informed molecular modeling of transporter-ligand interactions.

Comparisons of SERT pharmacology in native rat and human tissue preparations reveal consistent differences in antagonist sensitivities across species (8–10), results we have confirmed using the cloned rat and human SERTs heterologously expressed in HeLa cells (11). Thus, the tricyclic antidepressants, typified by imipramine, are more potent at human SERT, compared with rat SERT, in both [^{3}H]5-HT uptake and radioligand binding studies, although 5-HT, Na^+, and Cl^- recognition appears equivalent in the two species (11). Other antagonists, such as cocaine, fluoxetine, and paroxetine, show no SERT species selectivity. The substrate *d*-amphetamine displays the reverse sensitivity, compared with tricyclics, being more potent at rat than human SERT. Notably, rat and human SERT proteins bear limited sequence divergence, displaying 92% amino acid identity, which suggests that small domains or individual residues might determine species differences in drug potency. Using chimeras of rat and human SERTs, the carboxyl region of SERT distal to TMD11 (amino acid 531) was identified as the domain involved in the species selectivity of both imipramine and *d*-amphetamine (11). Because chimeras between SERT and the homologous human NET revealed little or no contribution of cytoplasmic amino or carboxyl termini to substrate and antagonist recognition (12, 13), we presumed that residues between amino acid 531 and the carboxyl terminus were likely to contribute to species differences in ligand binding.

In the present study, we use heterologous expression of parental and mutant SERT cDNAs in mammalian cells to identify the site responsible for increased potency of tricyclic antidepressants at human SERT. Specifically, we first confirmed the lack of involvement of the divergent carboxyl terminus in antidepressant recognition and then proceeded to mutate divergent residues between amino acid 531 and the carboxyl tail. Our strategy was to mutate divergent residues in rat SERT to their human SERT identity and test for an increase in the potency of tricyclic antidepressants, similar to that displayed by transfected human SERT. Candidate residues for tricyclic recognition would then be tested by conversion of the same residue in human SERT to its rat SERT identity, to test for a reciprocal loss of tricyclic potency. These studies implicated a single residue, F586, as imparting the higher affinity interactions of the tricyclic antidepressants with human SERT. No single amino acid was found to be responsible for species-variant *d*-amphetamine recognition. The contextual requirements for tricyclic recognition at amino acid 586 were further explored by mutation of the equivalent position in the tricyclic-sensitive human NET and the introduction of additional amino acids at the V586 position of rat SERT. Our findings confirm the utility of cross-species comparisons for identification of molecular determinants of neurotransmitter transporter antagonist binding sites.

Materials and Methods

[^{3}H]5-HT uptake assay in transfected cells. To determine the pharmacological profiles of cloned parental (6, 7) and mutant transporters, heterologous expression of SERTs or NETs was achieved with the recombinant T7 vaccinia virus expression system in HeLa cells (14). The transporter cDNAs had previously been cloned into the plasmids pBluescript SK or KS II(−) (6, 7) (for SERTs) or pcDNA3 (for NET), such that their start codons were downstream of the plasmid-encoded T7 RNA polymerase promoter. HeLa cells were maintained in Dulbecco's modified Eagle medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin, at 37° in a humidified 5% CO_2 incubator. To transfect cells for uptake assays, HeLa cells (100,000 cells/well) in 24-well culture dishes were infected with recombinant vaccinia virus VTF_{7-3} (15), which encodes T7 RNA polymerase, at 10 plaque-forming units/cell in OPTI-MEM I medium (GIBCO/BRL, Gaithersburg, MD) containing 55 μM β-mercaptoethanol. Thirty minutes after virus infection, the SERT cDNA constructs (100 ng/well) or NET cDNA constructs (500 ng/well) were introduced into the HeLa cells by liposome-mediated transfection (Lipofectin; GIBCO/BRL) at a ratio of 1 μg of DNA:3 μg of Lipofectin, mixed in OPTI-MEM I medium with 55 μM β-mercaptoethanol.

Six hours after transfection, cells were assayed for 5-HT transport activity with 20 nM [^{3}H]5-HT, 100 μM pargyline, and 100 μM L-ascorbate in KRH buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.18% glucose, 10 mM HEPES, pH 7.4), in triplicate, as described previously (6, 14). Because DA is a comparable but less expensive substrate than norepinephrine (16), 20 nM [^{3}H]DA with 100 μM pargyline, 100 μM L-ascorbate, and 10 μM U-0521 in KRH buffer was used in all NET experiments. Briefly, cells were preincubated in KRH buffer, with or without varying concentrations of uptake inhibitors, for 10 min at 37°, followed by the addition of ^{3}H-labeled substrate for 10 min at 37°. Uptake was terminated by three washes with ice-cold KRH buffer, cells were solubilized in 1 ml of liquid scintillant (Optiphase SuperMix; Wallac, Gaithersburg, MD), and the level of accumulated ^{3}H-labeled substrate in the well was determined directly with a Wallac MicroBeta plate counter. [^{3}H]5-HT uptake in HeLa cells transfected with pBluescript SK II(−) or [^{3}H]DA uptake in cells transfected with pcDNA3 was subtracted from the total uptake values to define specific uptake. Resulting data were plotted and IC_{50} or K_m values were obtained by using nonlinear least-squares curve fits (Kaleidagraph; Synergy Software, Reading, PA). K_i values (mean ± standard error) were determined after adjustments for substrate concentration, as described by Cheng and Prusoff (17). Mean data from at least three experiments were compared by using a two-sided Student's *t* test (GraphPAD InStat for MacIntosh, version 2.03; Intuitive Software for Science, San Diego, CA).

Rat/human SERT carboxyl-tail chimera. To subdivide clusters of species variant residues into those associated with TMD 12 or the carboxyl terminus, a chimeric protein was constructed that replaced the carboxyl tail of the rat SERT (amino acids 594–630) with the corresponding region from human SERT (Fig. 1). Using oligonucleotide primers encoding nucleotides 1794–1841 (sense) and 2146–2185 (antisense) of the human SERT cDNA, the 391-base pair region encoding the human SERT carboxyl tail was amplified with a polymerase chain reaction (95° for 1 min, 45° for 2 min, and 72° for 3 min, for 20 cycles, using Vent DNA polymerase). The sense primer was designed to maintain rat SERT identity throughout the TMD 12 region and also introduced a unique *Avr*II restriction site at position 1803, whereas the antisense primer introduced a *Xba*I restriction site at position 2155. Subsequently, the polymerase chain reaction-amplified human SERT carboxyl-tail fragment was digested with *Avr*II and *Xba*I, gel-purified, and ligated into the rat SERT cDNA (*Avr*II and *Xba*I fragment removed) using T4 DNA ligase. Dideoxy sequencing verified the cDNA construction for chimeric protein sequence.

Site-directed mutagenesis. To identify individual amino acids responsible for species selectivity of transporter ligands, the Stratagene Chameleon mutagenesis kit (Stratagene, La Jolla, CA) was used to introduce oligonucleotide-encoded point mutations in transporter cDNAs, focusing on the eight residues divergent between rat and human SERTs in the region found to be responsible for increased imipramine potency (amino acids 572–586) (Fig. 1). In separate

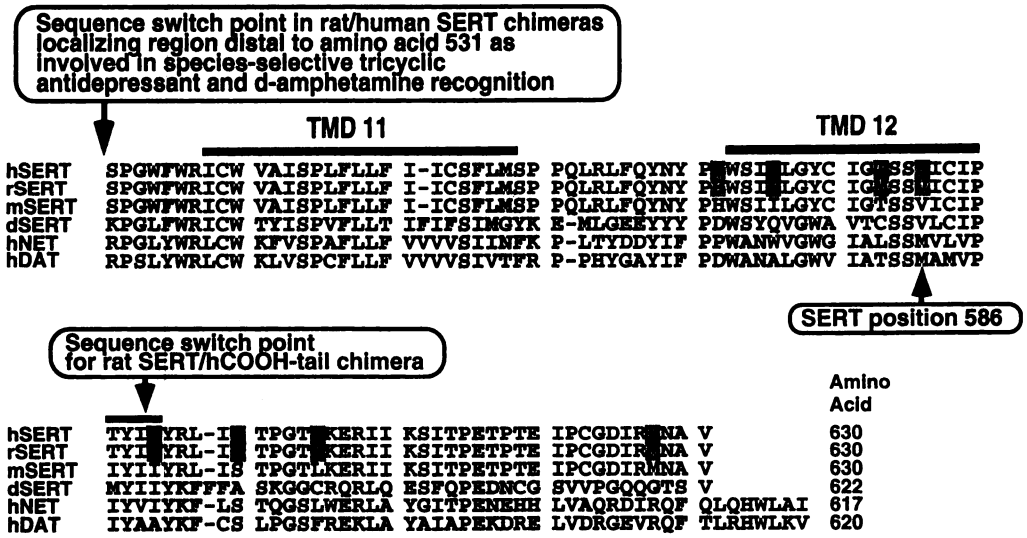

Fig. 1. Alignment of monoamine transporters in regions distal to amino acid 531. Alignment of human SERT and rat SERT shows no sequence differences up to the beginning of TMD 12. *Shaded residues*, residues that differ between rat and human SERTs. The sequence switch point for the rat/human SERT chimera with rat SERT containing the human SERT carboxyl tail (*rat SERT/hCOOH-tail*) is indicated. *hSERT*, human SERT; *rSERT*, rat SERT; *mSERT*, mouse SERT; *dSERT*, *D. melanogaster* SERT; *hNET*, human NET; *hDAT*, human DAT.

constructs, we produced rat SERT H572Y, V576I, M583T, V586F, V586Y, V586R, and V586D and the double-mutant M583T/V586F, human SERT F586V, and human NET M566F. Human NET M566 would be predicted to be equivalent to SERT position 586, based on sequence alignments (Fig. 1); however, we acknowledge the difficulty of confirming the equivalence of positions across transporter species. Dideoxy sequencing of mutated regions confirmed the presence of the mutations as the only coding sequence alterations in the cDNAs and the absence of additional mutations in the parental cDNA templates. For rat SERT mutants, *Aat*II/*Afl*II digestion fragments bearing the mutations were gel-purified and reinserted into the parental rat SERT plasmid (*Aat*II-*Afl*II fragment removed). Similarly, an *Apa*I fragment bearing the human SERT F586V mutation was substituted for the parental *Apa*I fragment, and human NET M566F was constructed from the *Ppu*MI and *Xba*I fragments of the parent and mutated cDNAs, respectively.

Materials. Dulbecco's modified Eagle medium was purchased from Fisher Scientific (Fair Lawn, NJ), fetal bovine serum from Hyclone (Logan, UT), and HeLa cells from the American Type Culture Collection (Rockville, MD). Trypsin, glutamine, penicillin, streptomycin, OPTI-MEM I medium, and Lipofectin were obtained from GIBCO/BRL, and cell culture plates from Falcon/Becton-Dickinson Labware (Mountain View, CA). Vaccinia virus-T7 RNA polymerase (VTF_{7-3}) was prepared from stocks provided by Dr. Bernard Moss, National Institute of Allergy and Infectious Diseases. [^{3}H]5-HT trifluoroacetate (~100 Ci/mmol) was purchased from Amersham Life Science (Clearbrook, IL). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA), Chameleon mutagenesis kit was purchased from Stratagene, and Optiphase SuperMix scintillation fluor was obtained from Wallac (Gaithersburg, MD). Cocaine hydrochloride was a gift from Dr. J. Justice, Emory University (Atlanta, GA), RTI-55 was a gift from Dr. John Boja, Department of Pharmacology, NEOUCOM (Rootstown, PA), and paroxetine was provided by Dr. Michael Owens, Emory University. All other drugs and materials were obtained from either Sigma Chemical (St. Louis, MO) or Fisher Scientific and were of the highest grade available.

Results

Evidence that the carboxyl terminus does not contribute to species selectivity for tricyclics or amphetamine. Our previous studies with rat and human SERT chimeras (11) established that the region distal to amino acid 531 was responsible for the species selectivity of the tricyclic antidepressants and *d*-amphetamine. Comparisons of the rat and human SERT amino acid sequences revealed that only eight amino acids are divergent between the two species in this region (Fig. 1). Based upon current hydrophobicity models for SERT topology (5–7), four of these residues would be proposed to be in or near TMD 12, whereas the remaining four amino acids would be located near or within an intracellularly localized carboxyl tail. To first establish whether residues responsible for characteristic species pharmacology were localized in the carboxyl tail or TMD 12 region, a rat/human SERT chimera was constructed that replaced the carboxyl tail of rat SERT (amino acids 594–630) with the equivalent domain from human SERT (Fig. 1). Compared with the parental rat and human SERTs, the rat SERT/human carboxyl-tail chimera exhibited imipramine and *d*-amphetamine potencies for inhibition of 5-HT uptake similar to those of the parental rat SERT (Fig. 2). In addition, cocaine displayed a potency for inhibition of 5-HT uptake that was equivalent for rat SERT, human SERT, and rat SERT/human carboxyl tail (577 ± 91, 457 ± 62, and 568 ± 99 nM, respectively) (data not shown), consistent with previous results showing that cocaine does not discriminate between rat and human SERTs. These results suggested that the carboxyl-tail region was not involved in mediating the differences in drug potencies observed for rat and human SERTs, and they focused our attention on the four residues in the TMD 12 region.

Site-directed mutagenesis implicating amino acid 586 in TMD 12 in tricyclic recognition. Using site-directed mutagenesis, we substituted human SERT amino acid identities into each of the four divergent positions in rat SERT proximal to the switch point in the rat SERT/human carboxyl-tail chimera. Thus, the following rat SERT mutations were constructed: H572Y, V576I, M583T, and V586F (Fig. 1). This initial strategy was chosen because it would test the power of a single amino acid substitution to increase tricyclic antidepressant potency at rat SERT to that of human SERT and would avoid a mutant screen based upon a loss in antagonist potency, which could result from nonspecific effects on protein conformation. Also, we chose to focus initially on mutation-induced shifts in rat SERT tricyclic sensitivities because of the greater magnitude of species differences, compared with *d*-amphetamine shifts in potency. As with the rat and human SERT chimeras (11), all rat SERT point mutants appeared to exhibit 5-HT transport activity

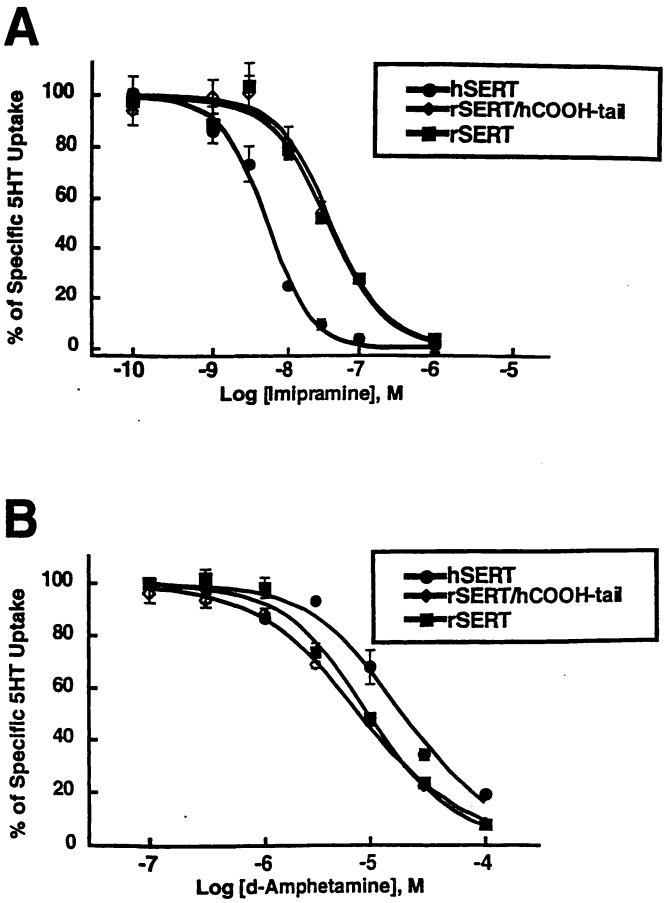

Fig. 2. Pharmacological characterization of the chimera consisting of rat SERT with the human SERT carboxyl tail. The sequence switch point for the chimera is indicated in Fig. 1. [^{3}H]5-HT uptake assays were performed on transiently transfected HeLa cells, as described in Materials and Methods, in the presence of 20 nM [^{3}H]5-HT. Nonspecific uptake was determined in HeLa cells transfected with the parent vector pBluescript SK II(−) and was subtracted from total values (control uptake values, 155 ± 37 cpm; five experiments). Data were plotted as percentage of specific 5-HT uptake. All data plotted represent means ± standard errors of triplicate determinations and are representative of three separate experiments. A, Evaluation of imipramine potency for rat SERT (*rSERT*), human SERT (*hSERT*), and rat SERT with the human SERT carboxyl tail (*rSERT/hCOOH-tail*). Increasing concentrations of imipramine were added 10 min before the addition of [^{3}H]5-HT. Apparent K_i values were as follows: human SERT, 4.6 ± 0.8 nM; rat SERT, 40 ± 4.0 nM; rat SERT with the human SERT carboxyl tail, 38 ± 0.5 nM. B, Evaluation of *d*-amphetamine potency for rat SERT, human SERT, and rat SERT with the human SERT carboxyl tail. Increasing concentrations of *d*-amphetamine were added simultaneously with the addition of [^{3}H]5-HT. Apparent K_i values were as follows: human SERT, 17,250 ± 450 nM; rat SERT, 8250 ± 1250 nM; rat SERT with the human SERT carboxyl tail, 8750 ± 850 nM. All K_i values represent means ± standard errors from the results of three separate experiments.

equivalent to that of the parental rat transporter (data not shown). We found that rat SERT H572Y, V576I, and M583T exhibited antagonist and substrate potencies similar to those of the parental rat SERT; thus, these residues could not account for the species selectivity for ligands (Table 1). In contrast, the V586F mutation resulted in an increase in imipramine potency (parental rat SERT, 31.9 ± 5.1 nM; V586F, 7.3 ± 0.7 nM) comparable to that observed for human SERT (4.6 ± 0.5 nM) (Table 1). Additionally, the shift in potency found for rat SERT V586F with imipramine was mirrored by effects on the other tricyclic antidepressants desipramine and nortriptyline (Table 1). The structurally distinct SERT antagonists cocaine and paroxetine, which originally showed no rat/human SERT selectivity, were not affected by the V586F mutation. Whereas *d*-amphetamine consistently showed a 3-fold selectivity for rat SERT over human SERT, no single amino acid substitution could be found in rat SERT that caused a reduction in *d*-amphetamine potency to show a human SERT-like phenotype. Because M583 and V586 should be in close proximity to one another on a TMD 12 α helix, we considered whether both of these residues might interact with each other and thus affect *d*-amphetamine potency. However, the double-mutant M583T/V586F exhibited no change in *d*-amphetamine potency, compared with the parental rat SERT, nor did the double mutation significantly augment the shift in tricyclic antidepressant potency observed with the V586F mutation alone.

Complementary changes in tricyclic potency induced for mutant human SERT but not human NET. If F586 is the primary determinant for tricyclic antidepressant species selectivity, then the complementary mutation to rat SERT V586F in human SERT (F586V) should cause a selective loss of tricyclic potency. Indeed, this substitution led to a decrease of imipramine potency essentially equivalent to that of wild-type rat SERT, with no effect on cocaine potency (Fig. 3) or *d*-amphetamine potency, compared with wild-type human SERT (data not shown). Additionally, the human SERT F586V mutant demonstrated a decreased potency for the other tricyclic compounds desipramine (human SERT, 69 ± 21 nM; F586V, 254 ± 88 nM; two experiments) (data not shown) and nortriptyline (human SERT, 57 ± 28 nM; F586V, 274 ± 41 nM; two experiments) (data not shown). The tricyclic antidepressants are also antagonists of the structurally related human NET, with the tertiary amine compounds such as imipramine exhibiting potency interactions more in line with rat SERT than human SERT (1). Therefore, to determine whether residues at the analogous position in human NET contribute to imipramine potency, we introduced a phenylalanine at the equivalent position in human NET, which normally has a methionine in this position (Fig. 1). Characterization of the human NET M566F mutant revealed no change in potency for any tricyclic compound or in DA transport kinetics, compared with the parental NET (Table 2). The NET M566F mutation did have a slight negative impact on cocaine and DA potency, of approximately 2-fold. These findings revealed that the effect of phenylalanine at this position is SERT-specific, suggesting that additional transporter-dependent interactions are involved in antagonist affinity.

Substitution of rat SERT V586 with tyrosine, aspartate, and arginine. To further explore the nature of the interaction of tricyclics with position 586, several different substitutions were introduced at this site in rat SERT and the mutants were tested for antagonist sensitivity. The potencies for paroxetine and the high affinity cocaine analogue RTI-55 were unaffected by any mutation at position 586 (Table 3). The aromatic rings of phenylalanine and tyrosine residues can contribute to nonpolar forces as well as such noncovalent polar forces as π-π or cation-π interactions. Surprisingly, substitution of V586 with a tyrosine, which differs from the aromatic amino acid phenylalanine only by the introduction of a *para*-hydroxyl group on the phenyl ring, negated the increase in imipramine sensitivity observed with the rat SERT V586F mutation (Table 3). To explore the

TABLE 1

Antagonist potencies for rat SERT mutants

K_i values are for the inhibition by various compounds of [^{3}H]5-HT uptake in HeLa cells transiently transfected with cloned human, rat, or mutant SERT. All mutations were introduced into rat SERT to substitute the identity of the equivalent position from human SERT, as described in Materials and Methods and illustrated in Fig. 1. Data represent means ± standard errors from results of three experiments performed in triplicate.

Drug	K_i						
	Rat SERT	Human SERT	Rat H572Y	Rat V576I	Rat M583T	Rat V586F	583/586[a]
	nM						
Imipramine	31.9 ± 5.1	4.6 ± 0.5[b]	26.2 ± 3.3	30.7 ± 14	31.0 ± 4.0	7.3 ± 0.7[b]	5.6 ± 1.4[b]
Desipramine	437 ± 100	54 ± 4.2[c]	ND[d]	ND	ND	143 ± 37[b]	157 ± 69[b]
Nortriptyline	220 ± 43	56 ± 20[c]	ND	ND	ND	135 ± 9[b]	90 ± 20[b]
Cocaine	496 ± 62	439 ± 78	386 ± 60	521 ± 49	700 ± 67	457 ± 17	ND
Paroxetine	0.2 ± 0.01	0.1 ± 0.01	0.2 ± 0.03	0.2 ± 0.04	0.1 ± 0.03	0.3 ± 0.08	ND
d-Amphetamine	5,430 ± 900	14,700 ± 2,200[b]	6,570 ± 1,110	6,290 ± 660	4,950 ± 670	5,330 ± 230	4,200 ± 700

[a] Double-mutant M583T/V586F.
[b] $p < 0.05$, compared with rat SERT value.
[c] Data from Barker *et al.* (11).
[d] ND, not determined.

potential role of ionic interactions in mediating the species-selective shifts in tricyclic potency, position 586 was also converted to either aspartate or arginine. Remarkably, the substitution V586D, which could introduce a negative charge at this position, increased imipramine potency to that observed for the rat SERT V586F and wild-type human SERTs. The effect of the V586D mutation was not exclusive for imipramine, because the other tricyclic compounds desipramine and nortriptyline exhibited increases in potency as well. In addition to increasing tricyclic antidepressant potency, the V586D mutation increased cocaine potency, although cocaine was insensitive to any other mutation at this position (Table 3). However, as mentioned above, the higher affinity cocaine analogue RTI-55 and the SERT-selective antagonist paroxetine were not affected by this mutation, nor were 5-HT transport kinetics altered, suggesting an absence of gross structural perturbations engendered by the V586D substitution. Introduction of the positively charged arginine at position 586 failed to shift antagonist potencies, although slight decreases in potencies were observed for the substrates *d*-amphetamine and 5-HT with this mutation.

Discussion

The present studies using site-directed mutagenesis of SERT cDNAs were designed to elucidate the molecular basis for the observed pharmacological differences identified in native tissues (8–10) and with cloned rat and human SERTs, as determined by both uptake and radioligand binding studies (11). Guided by our previous studies using rat/human SERT chimeras (11), single amino acid substitutions targeted to residues that were divergent between rat and human SERTs and were found in the region distal to residue 531 identified a single amino acid, F586, as being primarily responsible for the observed species differences for tricyclic antidepressant potency. Substitution at this position in rat SERT, V586, with the corresponding amino acid found in human SERT, F586, led to a rat SERT mutant (V586F) that exhibited human SERT phenotype with regard to tricyclic antidepressant potency. Likewise, the complementary mutation human SERT F586V displayed rat SERT-like imipramine potency, as would be expected if F586 mediates the higher potency interactions of imipramine with human SERT. Furthermore, we have presented data showing that no other single divergent amino acids in TMD 12 or residues in the carboxyl tail are involved in any alterations of ligand potency. Thus, a single amino acid variation between these two closely related transporters largely accounts for differences in tricyclic antidepressant potency. Our studies parallel cross-species comparisons of 5-HT (18) and tachykinin (19, 20) receptors that demonstrate the usefulness of cross-species chimeras to track ligand binding domains and that show that one or two amino acid substitutions among species homologues can have a profound impact on antagonist potency.

Regarding substrate interactions, we previously showed that *d*-amphetamine was more potent at rat SERT, compared with human SERT, and that this species selectivity tracked to the same domain as that involved in imipramine selectivity, whereas other amphetamine derivatives and 5-HT failed to exhibit species selectivity (11). The present studies fail to identify a single residue involved in the species differences observed for *d*-amphetamine. Particularly interesting, however, is the finding that the rat SERT V586F and human SERT F586V mutations can induce reciprocal shifts in imipramine potency while having no effects on *d*-amphetamine potency. This observation highlights the ability to discriminate between antagonist and substrate binding sites and suggests that the species-selective shift in *d*-amphetamine sensitivity is mediated in a more complex fashion than a single amino acid substitution contributes. The rat SERT V586R mutation does have a slight negative impact on both 5-HT and *d*-amphetamine potency. Further studies will be required to determine whether the TMD 12 region influences substrate potency through direct interactions with the substrate translocation pore or ligand binding pocket.

Analysis of the SERT point-mutants provides insight into the specificity of F586 for selectively increasing imipramine potency. None of the cross-species mutations or chimeras between rat and human SERT adversely affected any antagonist interactions or substrate transport kinetics with SERT, indicating that the mutations did not grossly perturb the conformational stability of SERT needed for uptake activity and ligand binding. Indeed, the rat SERT V586F mutation led to an increase in only tricyclic potency, whereas the human SERT F586V mutation induced a predictable loss of tricyclic potency but had no effects on the potency of other antagonists. These selective changes in tricyclic antidepressant K_i values occur without subsequent changes in 5-HT K_m

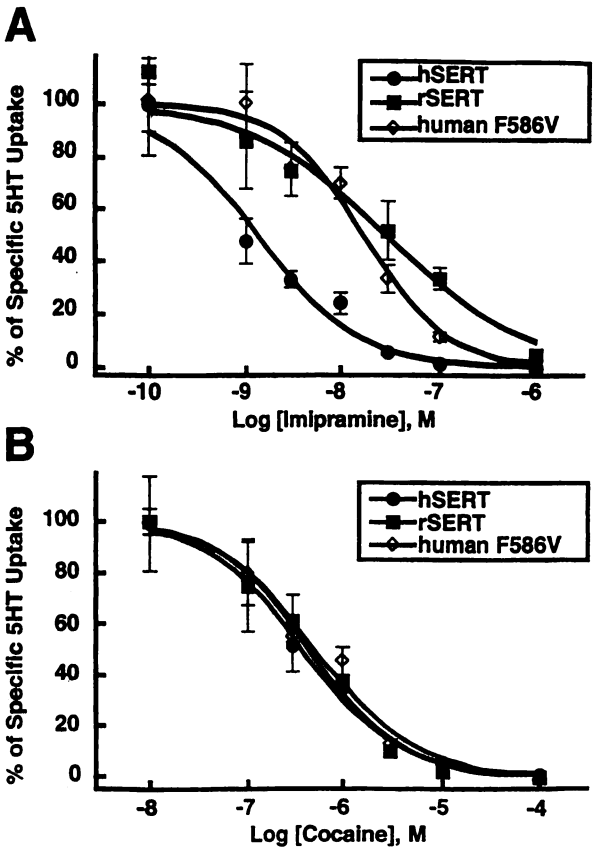

Fig. 3. Pharmacological characterization of mutant human SERT F586V, compared with wild-type rat and human SERTs. [^{3}H]5-HT uptake assays were performed with transiently transfected HeLa cells, as described in Materials and Methods, with increasing concentrations of antagonist added 10 min before the addition of 20 nM [^{3}H]5-HT. Nonspecific uptake was determined in HeLa cells transfected with the parent vector pBluescript SK II(−) and was subtracted from total values (control uptake values, 130 ± 12 cpm; five experiments). Data were plotted as percentage of specific 5-HT uptake. All data plotted represent means ± standard errors of triplicate determinations and are representative of three separate experiments. A, Evaluation of imipramine potency for rat SERT (*rSERT*), human SERT (*hSERT*), and mutant human SERT F586V (*human F586V*). Apparent K_i values were as follows: human SERT, 1.5 ± 0.2 nM; rat SERT, 26.3 ± 5.6 nM; human SERT F586V, 17.7 ± 1.7 nM. B, Evaluation of cocaine potency for rat SERT, human SERT, and human SERT F586V. Apparent K_i values were as follows: human SERT, 291 ± 49 nM; rat SERT, 449 ± 95 nM; human SERT F586V, 448 ± 139 nM. All K_i values represent means ± standard errors from the results of three separate experiments.

or V_{max} values, highlighting the lack of transport kinetic alterations with the mutant SERTs. These data are consistent with our rat/human SERT chimera studies, which showed that exchanging large domains from the two SERT homologues affects only tricyclic antidepressant and *d*-amphetamine potency, with no deleterious alterations in other SERT properties (11). Two other SERT species homologues, *Drosophila melanogaster* (21) and mouse (22) SERTs, also have a valine in position 586 (Fig. 1) and exhibit lower potency interactions with the tricyclic antidepressants. Thus, one interpretation of the present data would suggest that the presence of the valine at position 586 in wild-type rat SERT could create an unfavorable contact with imipramine through either repulsive forces or steric hindrance. Therefore, the removal of the valine, as in rat SERT V586F and the parental human SERT, would remove these negative effects

TABLE 2

Pharmacological characterization of human NET M566F mutant

K_i and K_m values for the inhibition by various compounds of [^{3}H]DA uptake in HeLa cells transiently transfected with the cloned human NET and M566F mutant were determined as described in Materials and Methods. Human NET position 566 aligns with SERT position 586, as illustrated in Fig. 1. Data represent means ± standard deviations from results of two experiments performed in triplicate. DA transport V_{max} values were 10.6 ± 6.2 × 10^{-18} and 6.6 ± 0.5 × 10^{-18} mol/min/cell for wild-type NET and the M566F mutant, respectively.

Drug	K_i or K_m	
	Human NET	NET M566F
	nM	
Imipramine	11.0 ± 3.3	15.7 ± 6.9
Amitriptyline	9.4 ± 1.8	6.6 ± 1.7
Desipramine	1.1 ± 0.1	0.7 ± 0.1
Cocaine	230 ± 5.7	430 ± 40*
DA	115 ± 43	290 ± 81

*$p < 0.05$.

and increase imipramine potency. However, results obtained with the rat SERT V586Y and V586R mutants would argue against this possibility, because these mutations did not increase imipramine potency, compared with that of the parental rat SERT. Indeed, the requirement for a phenylalanine at position 586 for increased tricyclic antidepressant potency is underscored by results obtained with the V586Y mutant, where the addition of a *para*-hydroxyl group to the phenyl ring appears to have disrupted a positive influence of phenylalanine on ligand potency, implying that the bulk of the aromatic side chain is not sufficient to produce these effects. Interestingly, the negatively charged, acidic amino acid aspartate is capable of reproducing the effects on imipramine potency observed with the phenylalanine substitution at this position, further suggesting that side chains at this position can either associate with ligands directly or associate with key SERT residues to establish a high affinity tricyclic antidepressant binding pocket.

Binding energy conversions indicate that the 5–10-fold shifts in potency observed across species are equivalent to a binding energy difference of approximately 1 kcal/mol, which is most indicative of van der Waals-type interactions (23). Because rat SERT V586F and V586D mutations displayed comparable shifts in imipramine potency, they should also alter binding energy by equivalent amounts, suggesting similar molecular interactions with the ligands. The phenyl rings of the tricyclic nucleus could interact with a phenylalanine via π-π stacking interactions (24), yielding the observed shifts in binding energy. However, the V586D mutant should not participate in this type of interaction, suggesting that the phenyl rings of the tricyclic structure are probably not directly involved. The amine group of imipramine could interact with the phenylalanine in a weakly polar fashion through amino-aromatic interactions, resulting in the observed shifts in potency (24). This interaction is not reproduced by the V586Y mutation, perhaps because the polarity of the hydroxyl group interferes with the amino-aromatic interaction by electron withdrawal from the phenyl ring or by physical displacement of the ring resulting from additional contacts with other SERT domains. Indeed, mutations of tachykinin receptors (25) and the nicotinic acetylcholine receptor (26) have shown that tyrosine does not always functionally substitute for phenylalanine. In the nicotinic acetylcholine receptor, conversion of Y93 to phenylalanine appears

TABLE 3

Pharmacological characterization of rat SERT position 586 mutants

K_i or K_m values for the inhibition by various compounds of [^{3}H]5-HT uptake in HeLa cells transiently transfected with the cloned human, rat, or mutant SERT were determined as described in Materials and Methods. 5-HT transport V_{max} values were as follows: wild-type rat SERT, 5.5 ± 1.4 × 10^{-18}; V586Y, 7.0 ± 2.7 × 10^{-18}; V586R, 3.7 ± 1.1 × 10^{-18}; V586D, 1.9 ± 0.5 × 10^{-18} mol/min/cell. Data represent means ± standard errors from results of three experiments performed in triplicate.

Drug	K_i or K_m					
	Human SERT[a]	Rat SERT	V586F[a]	V586Y	V586R	V586D
	nM					
Imipramine	4.6 ± 0.5	54 ± 9	7.3 ± 0.7	58 ± 22	74 ± 27	4.7 ± 0.6[b]
Desipramine	54 ± 4.2[c]	389 ± 107	143 ± 37	ND[d]	ND	82 ± 11[b]
Nortriptyline	56 ± 20[c]	203 ± 24	135 ± 9	ND	ND	73 ± 19[b]
Cocaine	346 ± 24	632 ± 170	457 ± 17	583 ± 129	703 ± 346	156 ± 36[b]
RTI-55	0.1 ± 0.04[c]	0.6 ± 0.1	ND	1.0 ± 0.2	0.9 ± 0.4	0.6 ± 0.1
Paroxetine	0.3 ± 0.09	0.3 ± 0.1	0.3 ± 0.08	0.3 ± 0.1	0.2 ± 0.09	0.2 ± 0.05
d-Amphetamine	14,700 ± 2,200	7,400 ± 800	5,330 ± 230	8,450 ± 1,650	16,250 ± 4,150[b]	9,300 ± 3,100
5-HT	499 ± 89[c]	418 ± 128	ND	504 ± 172	745 ± 90[b]	425 ± 45

[a] Unless otherwise indicated, human SERT and rat SERT V586F data were taken from Table 1 for comparison.
[b] $p < 0.05$, compared with rat SERT value.
[c] Data from Barker *et al.* (11).
[d] ND, not determined.

to cause a loss of hydrogen-bonding capacity with the agonist acetylcholine (26). However, substitution of Y198 with phenylalanine or other unnatural phenylalanine/tyrosine derivatives also causes a loss of acetylcholine potency, effects that suggest acetylcholine coordination through cation-π interactions (26). The amine group of imipramine could interact with the negatively charged aspartate; however, direct ionic bonding should provide a much larger increase in binding energy (23), resulting in a much larger increase in potency than what we observed unless the interactions of the amine are largely coordinated elsewhere in the SERT molecule. Consistent with the former idea, the amine group is critical for tricyclic antidepressant binding to SERTs (27); however, the shifts in potency observed across species are suggestive of only modulatory binding interactions. Thus, if the V586F and V586D mutations increase tricyclic antidepressant potency through associations with the alkylamine nitrogen, then these cation-π or cation-acid interactions assist only in coordination of ligand binding and probably do not constitute critical components of the tricyclic binding pocket. Similarly, Dougherty (28) has suggested that multiple coordinated cation-π interactions form the ligand binding pocket for many G protein-coupled receptors and ion channels. Conceivably, useful tests of these hypotheses could make use of a tricyclic analogue lacking the alkylamine chain; however, such compounds were unavailable to us, and their very low affinity for SERTs would likely reduce confidence in mutation effects (27).

Another possible interpretation of our results would be that F586 serves to stabilize human SERT in a conformation that has higher affinity for the tricyclic antidepressants, without having direct interactions with the antagonist itself. Both the aspartate and phenylalanine residues at position 586 could participate in intramolecular associations with residues in other domains of SERT possessing side-chain amino groups, such as lysine, arginine, glutamine, asparagine, and histidine. The phenylalanine could form favorable amino-aromatic interactions, and the aspartate could form a stronger ionic bond with a positively charged amine, thus creating an even more stable complex or dramatic conformational shift, possibly revealing sites that contribute binding energy not only to the tricyclics but also to cocaine, as revealed by the rat SERT V586D data. It is surmised that the high affinity cocaine analogue RTI-55 recruits additional major determinants for high affinity interactions with SERT and, hence, is not affected by the subtle nature of the V586D mutation. For the phenylalanine to form such interactions, however, the partner residue would have to lie 3.0–6.0 Å from the phenyl ring (24). Because one rotation on an α helix is approximately 5 Å, the interacting residue could exist within TMD 12, but no candidate residues exist this distance from position 586. Therefore, the interacting residue may reside in a more distal domain that exists in close spatial proximity to TMD 12 in the properly folded SERT tertiary structure. Clearly, a better understanding of the tertiary structure of SERT would assist in further exploration of this hypothesis. Relatively little is known regarding the structure of the active SERT molecule beyond the topology predicted from hydropathy analysis (6, 7); however, studies using antibodies targeted to the SERT carboxyl-tail region appear to confirm this intracellular localization of the domain (29).[1] Antipeptide antibodies raised against both putative intracellular and extracellular domains have also been used in topological studies of the homologous human NET, yielding results supportive of the original proposed topology of NET (30). Although these studies substantiate intracellular and extracellular domains, they do little to provide insight into the three-dimensional packing arrangements of TMDs most likely contributing the residues forming the antagonist binding pocket.

In the absence of X-ray crystallographic data, three-dimensional computer modeling can provide a starting point for structural analysis. Indeed, such a model has been generated for human DAT, which orients TMDs 1 and 7 near TMDs 8–12 for interactions with cocaine and DA (31). Studies using NET/DAT chimeras partially substantiate computer modeling, in that they implicate TMDs 1–3 as contributing primary determinants for substrate affinity, TMDs 5–8 as being involved in substrate translocation, and TMDs 9–11 as containing modulatory sites for substrate affinity (32–34). For antagonist binding, TMDs 5–8 have the primary influence on affinity and selectivity, whereas TMDs 1–3 contain second-

[1] S. Schroeter, unpublished observations.

ary determinants for antagonist interactions. Because these chimera studies fail to show tricyclic antidepressant interactions with the TMD 12 region, the interaction we describe for F586 may be a SERT-specific effect, consistent with our results for the human NET M566F mutation (see below). However, if F586 does not serve as a direct contact site for antidepressant binding, then this residue might interact with other residues in TMDs 1–3 or 5–8, regions implicated in antidepressant binding by the NET/DAT chimera studies. Such a model suggests that the binding pocket for transporter antagonists is formed by the complex interactions of multiple domains of the protein. In contrast to the studies described for NET and DAT, our data with the rat SERT V586D mutation clearly demonstrate that the TMD 12 domain can be altered to influence cocaine potency. Interestingly, photoaffinity-labeled cocaine analogues have been used to localize DAT antagonist binding sites to a region delineated by TMDs 4–12 (35), although clearly no predictions for involvement of specifically TMD 12 could be drawn from the latter study.

Our attempt to extend the conclusions regarding F586 to other members of the monoamine transporter gene family, in this case with the human NET mutation M566F, suggested that the effect of F586 is SERT-specific, in that NET M566F displayed no increase in tricyclic antidepressant potency. Based on the greater potency of secondary amine tricyclic compounds at NET than at SERT, with the tertiary amine compounds showing the reverse selectivity, and the seemingly different conformational requirements for NET and SERT antagonists (27), the absence of effect observed for the NET M566F mutation could result from imipramine lacking interactions altogether with position 586 in human NET. This finding highlights the greater sequence divergence, particularly in the TMD 12 region (Fig. 1), between NET and SERT, further suggesting that NET has unique contact sites for interactions with tricyclic compounds not shared with SERT. The recruitment of higher potency interactions of cocaine with the V586D mutation indicates that the TMD 12 region can influence other ligands as well. However, the lack of mutation-induced effects on the nontricyclic paroxetine and on the substrate 5-HT clearly demonstrates that antagonist and substrate binding domains can be discriminated from one another. In conclusion, we present data identifying the residue involved in the species selectivity of the tricyclic antidepressants. Future studies using additional molecular and biophysical approaches should provide insight into the specific function that F586 serves to influence ligand potency.

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Send reprint requests to: Randy D. Blakely, Ph.D., Department of Pharmacology and Center for Molecular Neuroscience, Vanderbilt University School of Medicine, MRB II, Room 419, Nashville, TN 37232-6600. E-mail: randy.blakely@mcmail.vanderbilt.edu