0026-895X/94/050799-09$03.00/0
Copyright © by The American Society for Pharmacology and Experimental Therapeutics
All rights of reproduction in any form reserved.
MOLECULAR PHARMACOLOGY, 46:799-807, 1994.

Chimeric Human and Rat Serotonin Transporters Reveal Domains Involved in Recognition of Transporter Ligands

ERIC L. BARKER, HEATHER L. KIMMEL, and RANDY D. BLAKELY

Department of Anatomy and Cell Biology (E.L.B., R.D.B.) and the Graduate Program in Neuroscience (H.L.K.), Emory University School of Medicine, Atlanta, Georgia 30322

Received June 28, 1994; Accepted August 29, 1994

SUMMARY

The serotonin transporter (SERT) is a target for many clinically significant drugs, such as cocaine, amphetamine, and antidepressants. The relationship between the structure of SERT and the binding of substrates and antagonists is virtually unknown, despite a large body of data describing the structure-activity relationships of transporter ligands. The cloning of multiple species homologs of SERT affords a unique opportunity for molecular comparisons to identify potential domains and residues involved in ligand recognition. We have conducted pharmacological comparisons of the cloned rat and human SERTs in transiently transfected HeLa cells. Serotonin uptake and radioligand binding assays revealed that rat and human SERTs show different sensitivities to some but not all transporter ligands; most tricyclic antidepressants were significantly more potent at the human SERT, relative to rat SERT, whereas *d*-amphetamine was a more potent inhibitor of rat SERT. Several other ligands, such as fluoxetine, paroxetine, (+)-methylenedioxymethamphetamine, cocaine, and the substrate 5-hydroxytryptamine, showed no significant species selectivity. Cross-species chimeras between rat and human SERTs were constructed to track the species-specific pharmacologies through the SERT molecule. These chimeric SERTs were expressed in HeLa cells and transported serotonin similarly to parental SERTs. Using these chimeras, we have isolated a region distal to amino acid 532 that imparts species preferences for both the tricyclic imipramine and *d*-amphetamine. Our results support the prediction of distinct binding sites for SERT ligands and implicate a restricted region in or near putative transmembrane domain 12 of the transporter as being involved in both substrate and antagonist recognition.

The SERT is responsible for synaptic and plasma clearance of 5-HT and is a major target for many antidepressants, as well as important drugs of abuse such as cocaine and amphetamine. The cloning of the rat (1, 2), human (3), mouse (4), and *Drosophila* (5, 6) SERTs has revealed the transporter to be a member of the large r γ-aminobutyric acid transporter/NET gene family of transport proteins (7). Like other transporter homologs, the SERT amino acid sequence predicts 12 putative TMDs, a large extracellular loop between TMD 3 and TMD 4 with multiple sites for *N*-linked glycosylation, and multiple consensus sequences in the intracellular domains for phosphorylation by protein kinases. The human and rat SERTs are both 630-amino acid polypeptides, with 92% overall sequence homology (3). Upon heterologous expression in mammalian cells, the cloned SERTs confer Na^+- and Cl^--dependent high affinity uptake of 5-HT that is sensitive to inhibition by SERT antagonists, including imipramine, paroxetine, and cocaine, with rank orders of potencies similar to those observed in native membrane preparations (8–11). Extracellular Na^+ and Cl^- concentrations govern the binding of many transport inhibitors and provide the ion gradients necessary for active 5-HT uptake (12–14).

Despite a large body of information describing the pharmacology of SERTs and a knowledge of SERT primary amino acid sequences, little data are available relating SERT structure to function and drug recognition. One approach that has been useful in identifying ligand binding domains of G protein-coupled receptors and ion channels has been the exploitation of species differences in antagonist potencies, in which cross-species chimeras and site-directed mutagenesis can be used to localize specificity-defining domains and residues, respectively (for review, see Ref. 15). Those studies demonstrate that as little as a single amino acid substitution across species homologs can have a profound impact on antagonist potency, thus identifying potential residues that may directly serve as contact points in ligand binding domains (16, 17). In this regard, species differences in the pharmacologies of endogenous rat and human

This work was supported by National Institutes of Health Grant DA07390 (R.D.B.), the Mallinkrodt Foundation (R.D.B.), and the Emory University Center for Neurological Sciences (E.L.B.).

ABBREVIATIONS: SERT, serotonin transporter; 5-HT, 5-hydroxytryptamine (serotonin); TMD, transmembrane domain; KRH buffer, Krebs-Ringer-HEPES buffer; (+)-MDMA, (+)-methylenedioxymethamphetamine; NET, norepinephrine transporter; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

SERTs have been reported (10, 18–20). For example, the tricyclic antidepressants imipramine and clomipramine were found to be more potent at human platelet SERTs, relative to rat preparations (10). However, those studies were conducted with tissue preparations, where the influences of other ligand binding sites and tissue variability are difficult to control. Potentially, platelet SERT proteins also might be modified differently in the two species, leading to the altered pharmacology observed. Alternatively, differences in pharmacological profiles may reflect inherent variations in the amino acid sequences of rat (1, 2) and human (3) SERTs. These questions can be addressed by heterologous expression of the cloned SERT cDNAs in the same mammalian cell context. After expression of cloned rat and human SERTs in HeLa cells, we verified that both transporters bear intrinsic and distinct sensitivities to certain transporter substrates and antagonists, similar to those reported in native membrane preparations. The tricyclic antidepressants typified by imipramine were more potent at the cloned human SERT, compared with the rat SERT, whereas *d*-amphetamine, a SERT substrate, was more potent at the rat SERT, relative to the human SERT. The nontricyclic compounds like paroxetine, fluoxetine, and cocaine, as well as 5-HT, showed no species preferences. Differential imipramine sensitivities were also observed in radioligand binding assays, where modulatory ionic binding sites were eliminated as a major contributor to species-specific characteristics. Finally, cross-species chimeras between rat and human SERTs were used to identify a common domain involved in the high affinity interactions of both imipramine and *d*-amphetamine.

Materials and Methods

[^{3}H]5-HT uptake assay in transfected cells. To directly compare the pharmacological profiles of the cloned human (3) and rat (1) SERTs, heterologous expression of the transporters was achieved using the recombinant vaccinia virus T7 expression system in HeLa cells (21). The human SERT and rat SERT cDNAs had previously been cloned into the plasmids pBluescript KS II(−) (3) and pBluescript SK II(−) (1), respectively, 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, L-glutamine (2 mM), and penicillin (100 units/ml) streptomycin (100 μg/ml), at 37° in a humidified 5% CO_2 incubator. To transfect cells for uptake assays, HeLa cells (50,000 cells/well in 48-well culture dishes) were infected with recombinant vaccinia virus vTF_{7-3} (22), which encodes T7 RNA polymerase, at 10 plaque-forming units/cell in OPTI-MEM I medium containing 55 μM β-mercaptoethanol. Thirty minutes after virus infection, the human SERT or rat SERT cDNA constructs (50 ng/well) were introduced into the HeLa cells by liposome-mediated transfection (Lipofectin), at a ratio of 1 μg of DNA:3 μg of Lipofectin (mixed in OPTI-MEM I medium containing 55 μM β-mercaptoethanol).

Six hours after transfection, 5-HT transport assays 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) were performed in triplicate, as described previously (1, 21). 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]5-HT for 10 min at 37°. Uptake was terminated by three washes with ice-cold KRH buffer, cells were solubilized with 1% SDS, and the level of accumulated [^{3}H]5-HT was determined by liquid scintillation counting. [^{3}H]5-HT uptake in HeLa cells transfected with pBluescript SK II(−) was subtracted from the data to define specific uptake. Resulting data were plotted and IC_{50} values were obtained using nonlinear least-squares curve fits (Kaleidagraph; Synergy Software) to the four-parameter logistic equation. K_i values were determined after adjustments for substrate concentration, as described by Cheng and Prusoff (23).

SERT chimera constructions. Chimeras between rat and human SERTs were constructed by exploiting common restriction endonuclease sites found in the two cDNAs. Chimera $H_{1-362}R_{363-630}$ is composed of human SERT cDNA encoding amino acids 1–362 and rat SERT cDNA encoding amino acids 363–630. The resulting chimera contains the amino terminus and TMDs 1–6 of human SERT and TMDs 7–12 and the carboxyl terminus of rat SERT. This chimera was constructed by digesting human SERT/pBluescript KS II(−) with *Xba*I and rat SERT/pBluescript SK II(−) with *Kpn*I. After these sites were blunted with Klenow fragment of DNA polymerase, the two linearized fragments were digested with *Bst*EII and the resulting human SERT *Bst*EII fragment, encoding amino acids 1–362, was substituted for the corresponding rat SERT fragment by ligation. Chimera $R_{1-272}H_{273-630}$ is composed of rat SERT cDNA encoding amino acids 1–272 and human SERT cDNA encoding amino acids 273–630. The resulting chimera contains the amino terminus and TMDs 1–4 of rat SERT and TMDs 5–12 and the carboxyl terminus of human SERT. This chimera was constructed by swapping restriction fragments generated by digestion of the parental rat SERT and human SERT plasmids with *Bsa*HI. Chimera $H_{1-362}R_{363-531}H_{532-630}$ was created by substituting a *Sma*I restriction fragment from the parental human SERT cDNA for the *Sma*I fragment of chimera $H_{1-362}R_{363-630}$. The resulting chimera contains the amino terminus and TMDs 1–6 of human SERT, TMDs 5–10 of rat SERT, and TMDs 11 and 12 and the carboxyl terminus of human SERT. The chimeric constructs were transiently expressed in HeLa cells and used in 5-HT uptake assays, to compare their pharmacological profiles with those of the parental transporters, as described above.

Immunoprecipitations. Immunoprecipitations of SERT and chimeric transporter proteins were used to directly verify synthesis of the transporter proteins in transfected cells. The polyclonal SERT-specific antibody S365 (24) is targeted to the absolutely conserved epitope EMRNEDVSEVAKDA present in amino acids 388–401 of rat and human SERT and thus can be used to confirm synthesis of both parental and chimeric transporters.

For immunoprecipitations, ~500,000 HeLa cells/well were transiently transfected with rat SERT, human SERT, or rat/human chimeric cDNAs as described above, followed 4 hr later by washing with prewarmed methionine/cysteine-free medium and incubation with Trans^{35}S label (50 μCi/ml) in methionine/cysteine-free medium for 3 hr 37°. Labeled medium was removed, cells were washed with ice-cold phosphate-buffered saline, and cells were solubilized in ice-cold RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) supplemented with 1 mg/ml soybean trypsin inhibitor, 1 mM *o*-phenanthroline, 1 μg/ml leupeptin, 1 mM iodoacetamide, 1 μM pepstatin A, and 250 μM phenylmethylsulfonyl fluoride. Solubilized cell extracts were centrifuged at 20,000 × *g* for 10 min at 4°, followed by protein determination in supernatants using the detergent-compatible protein assay (Bio-Rad), with bovine serum albumin as the standard. Protein A-Sepharose beads were prepared as follows. Nonspecific binding was blocked by incubation with unlabeled native cell extracts (1 hr, 22°), followed by three washes in RIPA buffer. Beads were resuspended in RIPA buffer to a final concentration of 30 mg/ml and were incubated with the labeled cell extracts (1 hr, 22°) to further preclear any nonspecific protein binding. Labeled cell extracts were recovered by centrifugation of the beads, serum (25 μl) was added to the extracts, and samples were incubated (1 hr, 22°) with continuous mixing. Blocked Protein A-Sepharose beads were added to labeled cell extract/serum mixtures and incubated overnight at 4°, with continuous mixing. The beads were washed three times with RIPA buffer and bound protein was eluted into 100 μl of Laemmli sample buffer for electrophoresis on 8% SDS-PAGE gels. Gels were soaked in 1 M sodium salicylate, dried, and exposed to X-ray film at −80°.

[^{3}H]Imipramine binding. To prepare crude membranes, HeLa cells were transfected with SERT cDNAs as described for transport assays, except that cells were plated on 150-mm culture dishes and cells were used 12–16 hr after transfection to maximize SERT expression. Cells were washed with phosphate-buffered saline, incubated in ice-cold hypotonic buffer (50 mM Tris·HCl, pH 7.4, 100 mM NaCl), detached from the plates using a cell scraper, and centrifuged for 20 min at 27,000 × *g*. The resulting pellet was resuspended with a Brinkmann Polytron homogenizer (5 sec, 20,000 rpm) and centrifuged for an additional 20 min at 27,000 × *g*. The pellet was suspended in KRH buffer, homogenized with the Polytron homogenizer (10 sec, 20,000 rpm), and used in binding assays. Saturation binding was determined in duplicate using 5–15 nM [^{3}H]imipramine and increasing concentrations of unlabeled imipramine, with 1 μM paroxetine being used to define nonspecific binding. Assay tubes were incubated for 30 min at 22°, and assay mixtures were filtered, using a Brandel harvester, through Schleicher and Schuell no. 32 glass fiber filters that had been soaked in 0.5% polyethylenimine. Each tube contained 100 μg of protein as determined by the Bradford method (Bio-Rad), using bovine serum albumin as the standard.

The Na^+ dependence of [^{3}H]imipramine binding was assessed in KRH buffer using isotonic replacement of NaCl with LiCl, whereas the Cl^- dependence was determined in KRH buffer with Cl^- salts replaced by sodium gluconate, potassium gluconate, and $Ca(NO_3)_2$ at molarities equivalent to those in regular KRH buffer. All other binding parameters were held constant.

Materials. Dulbecco's modified Eagle medium was purchased from Fisher Scientific, fetal bovine serum from Hyclone, and HeLa cells from the American Type Culture Collection. Trypsin, glutamine, penicillin, streptomycin, OptiMEM I medium, and Lipofectin were obtained from GIBCO/BRL, and cell culture plates were from Falcon/Becton-Dickinson Labware. Vaccinia virus T7 RNA polymerase (vTF_{7-3}) was provided by Dr. Bernard Moss, National Institute of Allergy and Infectious Diseases. S365 antibody was produced in female New Zealand white rabbits maintained by Spring Valley Labs. SDS-PAGE molecular weight standards and SDS-PAGE reagents were from Bio-Rad, and Tran^{35}S label (1131 Ci/mmol) and methionine/cysteine-deficient medium were obtained from ICN; [G-^{3}H]5-HT creatinine sulfate (8.6 Ci/mmol) and [*benzene ring*-^{3}H(N)]imipramine hydrochloride (48.7 Ci/mmol) were purchased from New England Nuclear. Protein A-Sepharose was from Pharmacia. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs; EcoScint H scintillation fluor was obtained from National Diagnostics. (+)-MDMA was a gift from Dr. Gary Rudnick, Yale University; cocaine hydrochloride was a gift from Dr. J. Justice, Emory University; RTI-55 was a gift from Dr. John Boja, National Institute on Drug Abuse; *d*-amphetamine, *p*-chloroamphetamine, fenfluramine, maprotiline, sertraline, and quipazine were donated by Dr. Michael Owens, Emory University. All other drugs and materials were obtained from either Sigma Chemical Co. or Fisher Scientific and were of the highest grade possible.

Results

Pharmacological comparisons of rat and human SERTs. Using HeLa cells transiently expressing either the cloned human or rat SERT, we investigated the sensitivity of SERT species variants to inhibition by various substrates and antagonists (Table 1). The substrate 5-HT showed similar K_m values of 629 nM and 499 nM for rat and human SERTs, respectively. Among amphetamine derivatives known to be additional substrates for SERTs (25, 26), only *d*-amphetamine exhibited consistent species selectivity, being ~3-fold more potent at inhibiting 5-HT uptake at rat SERT versus human SERT, whereas *p*-chloroamphetamine and fenfluramine showed no marked differences in potencies across species.

Comparison of antagonist potencies for uptake inhibition at rat and human SERTs revealed that the heterocyclic antagonists, including cocaine, paroxetine, fluoxetine, sertraline, and citalopram, possessed little if any selectivity for either species variant. In contrast, human SERT was found to be significantly more sensitive to inhibition by the tricyclic antidepressant compounds clomipramine (0.8 nM versus 3.9 nM), imipramine (8.2 nM versus 46 nM), amitriptyline (8.4 nM versus 53 nM), desipramine (54 nM versus 209 nM), and protriptyline (268 nM versus 471 nM), relative to rat SERT. Interestingly, the tricyclic secondary amine nortriptyline exhibited no significant species selectivity. To verify that tricyclic compounds like imipramine possessed greater affinity for human SERT, compared with rat SERT, we assessed [^{3}H]imipramine binding in membranes prepared from cells expressing either human or rat SERT. Saturation binding curves fit to the Hill equation revealed B_{max} values that differed for each clone (human SERT, 5.1 ± 2.0 pmol/mg of protein; rat SERT, 9.5 ± 4.7 pmol/mg of protein; data not shown) in a manner consistent with 5-HT uptake measurements. Consistent with its differential potency in inhibition of 5-HT uptake, [^{3}H]imipramine exhibited significantly greater affinity for the human SERT (K_d = 3.2 ± 1.3 nM), compared with the rat SERT (K_d = 90 ± 31 nM).

Ion dependence of imipramine binding in human and rat SERTs. Because the binding of many antagonists, including imipramine, is Na^+ and Cl^- dependent (12, 13), the observed species differences in tricyclic antidepressant potencies could reflect different ionic sensitivities of rat and human SERTs, resulting in altered antagonist potencies when tested in a buffer of fixed ion composition. To rule out this possibility, we determined the K_d of imipramine in saturation binding experiments with various concentrations of either Na^+ (Fig. 1A) or Cl^- (Fig. 1B). Increasing Na^+ concentrations over a range from 50 mM to 300 mM led to increases in the affinity of imipramine for both rat SERT (K_d range, 10–102 nM) and human SERT (K_d range, 0.25–17 nM), with response curves that were similarly shaped when they were scaled to account for the observed differences in affinity. Salt concentrations higher than 300 mM were not used, to avoid nonspecific effects on ligand binding (12). The response curves for the affinity of imipramine for both clones displayed Hill coefficients greater than 1 (human, 3.6 ± 1.7; rat, 2.6 ± 0.5), using nonlinear curve fitting. Due to the lack of saturation in this analysis, these Hill coefficient values should be regarded as estimates, although they are consistent with data from Humphreys *et al.* (12). When the Na^+ concentration was fixed at 300 mM, the affinity of imipramine for both rat SERT (K_d range, 6.4–390 nM) and human SERT (K_d range, 0.2–10 nM) increased with buffer Cl^- concentration (Fig. 1B). Unlike the Na^+ sensitivity of imipramine binding, the Cl^- effect on the affinity of imipramine approached a maximum by 300 mM, with curves yielding Hill coefficients near 1 (human, 1.6 ± 0.4; rat, 1.1 ± 0.2), which were not significantly different from one another.

Identification of ligand recognition domains by using SERT chimeras. Chimeras between rat and human SERTs were used to distinguish transporter domains responsible for mediating the observed species-specific pharmacologies. Three chimeric transporters were created, $R_{1-272}H_{273-630}$, $H_{1-362}R_{363-630}$, and $H_{1-362}R_{363-531}H_{532-630}$. The SERT-specific antibody S365 immunoprecipitated parental and chimeric SERTs from metabolically labeled HeLa cells, verifying appropriate synthesis

TABLE 1

Antagonist and substrate affinities for SERTs

K_i and 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 chimeric SERT are shown. Drugs are grouped according to chemical classification. Data represent means ± standard errors from curve fits for competition curves with six to nine determinations for each concentration. Means were compared with rat SERT values using a two-sided Student t test (GraphPAD InStat version 3.0; Intuitive Software for Science).

Drug	K_i or K_m				
	Rat SERT	Human SERT	$R_{1-272}H_{273-630}$	$H_{1-362}R_{363-630}$	$H_{1-362}R_{363-531}H_{532-630}$
			nM		
5-HT	629 ± 116	499 ± 89	380 ± 44	619 ± 63	520 ± 110
Imipramine	46 ± 9.3	8.2 ± 3.4[a]	6.7 ± 1.2[a]	44 ± 9.1	7.6 ± 1.3[a]
Clomipramine	3.9 ± 0.8	0.8 ± 0.05[a]	1.1 ± 0.1[a]	3.8 ± 1.1	ND[b]
Desipramine	209 ± 45	54 ± 4.2[a]	120 ± 21	206 ± 33	ND
Amitriptyline	53 ± 17	8.4 ± 3.4[a]	7.1 ± 2.8[a]	33 ± 8.2	ND
Protriptyline	471 ± 48	268 ± 31[a]	189 ± 44[a]	327 ± 81	ND
Nortriptyline	117 ± 48	56 ± 20	ND	ND	ND
d-amphetamine	11,800 ± 400	30,700 ± 3,700[a]	29,800 ± 3,600[a]	11,300 ± 1,200	33,000 ± 4,900[a]
(+)-MDMA	790 ± 58	1,200 ± 140[a]	900 ± 39	820 ± 93	ND
Fenfluramine	1,000 ± 100	1,200 ± 140	1,003 ± 52	1,015 ± 700	ND
p-Chloroamphetamine	351 ± 47	685 ± 203	ND	ND	ND
Cocaine	540 ± 47	611 ± 66	496 ± 27	330 ± 18[a]	ND
RTI-55	0.26 ± 0.12	0.11 ± 0.04	ND	ND	ND
Paroxetine	0.09 ± 0.03	0.05 ± 0.01	0.03 ± 0.02	0.09 ± 0.01	ND
Citalopram	4.9 ± 1.1	4.7 ± 0.7	ND	ND	ND
Maprotiline	12,800 ± 5,000	6,700 ± 720	ND	ND	ND
Fluoxetine	7.3 ± 3.3	3.1 ± 0.6	ND	ND	ND
Sertraline	0.9 ± 0.2	1.3 ± 0.4	ND	ND	ND
Quipazine	26 ± 3.7	25 ± 3.7	ND	ND	ND

[a] $P < 0.05$, compared with rat SERT value.
[b] ND, not determined.

of the transporter proteins (Fig. 2). Chimeric transporters migrated with single major bands at ~60 kDa, as did the parental rat and human SERT proteins, consistent with previous findings on SERT mobility on SDS-PAGE (24).

To establish appropriate functional expression of chimeric SERTs, chimeras $R_{1-272}H_{273-630}$ and $H_{1-362}R_{363-630}$ were compared with rat and human SERTs for [^{3}H]5-HT substrate-velocity kinetics. These studies revealed that both chimeras transported 5-HT with equivalent K_m values (Table 1) and similar V_{max} values (rat SERT, 8.2 ± 0.3 × 10^{-18}; human SERT, 8.5 ± 0.2 × 10^{-18}; $H_{1-362}R_{363-630}$, 7.3 ± 0.2 × 10^{-18}; $R_{1-272}H_{273-630}$, 1.8 ± 0.6 × 10^{-18} mol/min/cell).

The pharmacological profiles of the rat/human SERT chimeras were determined in 5-HT uptake assays conducted in parallel with assays of cloned parental SERTs (Table 1). Paroxetine and fenfluramine, which exhibited no species preferences in our initial comparisons, similarly displayed no pharmacological selectivity for either chimera; however, imipramine inhibited 5-HT uptake at chimera $H_{1-362}R_{363-630}$ with a potency like that observed for rat SERT (Fig. 3A), whereas chimera $R_{1-272}H_{273-630}$ showed an imipramine sensitivity identical to that of human SERT (Fig. 3B). Similarly, the K_d values of imipramine for the two chimeras, as determined by saturation radioligand binding experiments, were 120 ± 53 nM for $H_{1-362}R_{363-630}$ and 4.0 ± 0.8 nM for $R_{1-272}H_{273-630}$, values consistent with the results of the uptake inhibition studies and similar to data from equilibrium imipramine binding to the parental rat (90 nM) and human (3 nM) SERTs, respectively.

Chimeric SERTs were then assayed for sensitivity to inhibition by *d*-amphetamine, which was found previously to be more potent at rat SERT, compared with human SERT. Similarly to the imipramine experiments, chimera $H_{1-362}R_{363-630}$ exhibited a rat SERT-like potency for *d*-amphetamine (Fig. 4A), whereas chimera $R_{1-272}H_{273-630}$ displayed a K_i for *d*-amphetamine comparable to that of human SERT (Fig. 4B). Like *d*-amphetamine, (+)-MDMA was also found to be less potent at human SERT, compared with rat SERT (Table 1); however, this more marginal discrimination was not evident in tests of cross-species chimeras.

To further isolate the region of SERT involved in the high affinity interactions of inhibitors with the SERT species homologs, chimera $H_{1-362}R_{363-531}H_{532-630}$ was constructed and evaluated for imipramine and *d*-amphetamine sensitivity. This chimera differs from the previously described chimera $H_{1-362}R_{363-630}$ only by the substitution of human amino acids 532–630, which include TMDs 11 and 12 and the carboxyl terminus, for the complementary rat SERT sequence. When expressed in HeLa cells, chimera $H_{1-362}R_{363-531}H_{532-630}$ conferred high affinity 5-HT uptake (V_{max} = 6.8 ± 0.4 × 10^{-18} mol/min/cell, K_m = 520 ± 110 nM) similar to that of both parental SERTs. Whereas chimera $H_{1-362}R_{363-630}$ had a pharmacology similar to that of the parental rat SERT, chimera $H_{1-362}R_{363-531}H_{532-630}$ displayed imipramine (Fig. 3C) and *d*-amphetamine (Fig. 4C) sensitivities equivalent to those displayed by heterologously expressed human SERT.

Discussion

Through the transfection of cloned rat and human SERT cDNAs into a single mammalian cell expression system, we have demonstrated an intrinsic species selectivity in the pharmacological profiles of rodent and human SERTs. Our data demonstrate that several tricyclic antidepressants have greater potency for the human SERT, compared with the rat SERT. Interestingly, the tricyclic nortriptyline showed no species differences with regard to potency for inhibiting 5-HT uptake. Conversely, *d*-amphetamine exhibited greater potency for rat

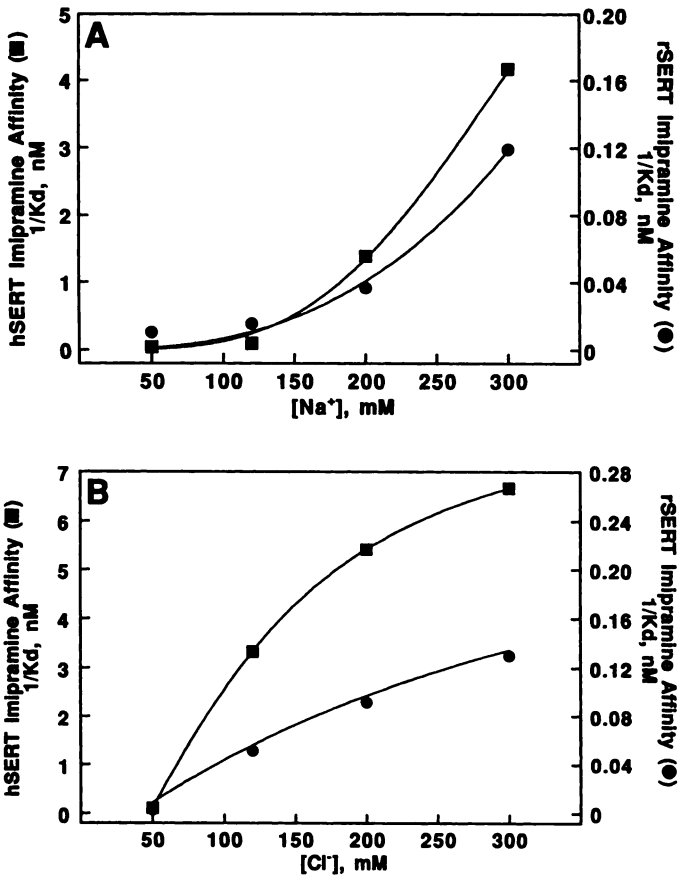

Fig. 1. Ion dependence of imipramine binding to rat SERT (*rSERT*) and human SERT (*hSERT*). A, Imipramine affinity for rat and human SERTs in the presence of increasing Na^+ concentrations, at a fixed Cl^- concentration of 300 mM. [^{3}H]Imipramine saturation binding was performed and the imipramine K_d was determined at each Na^+ concentration, as described in Materials and Methods. Affinity was defined as $1/K_d$. The average B_{max} value was 4.4 ± 0.8 pmol/mg of protein for human SERT and 8.9 ± 0.4 pmol/mg of protein for rat SERT (data not shown). Curves were fit to the Hill equation, resulting in estimated Hill coefficients of 2.6 ± 0.5 and 3.6 ± 1.7 for rat and human SERTs, respectively. B, Imipramine affinity for rat and human SERTs in the presence of increasing Cl^- concentrations, at a fixed Na^+ concentration of 300 mM. Average B_{max} values for these experiments were 5.7 ± 0.9 pmol/mg of protein for human SERT and 7.7 ± 0.5 pmol/mg of protein for rat SERT (data not shown). Curves were plotted using the Hill equation, yielding estimated Hill coefficients of 1.6 ± 0.4 and 1.1 ± 0.2 for human and rat SERTs, respectively. Data represent means from two separate experiments performed in duplicate, with an associated error of <10%. Note that the scale for the human SERT *y*-axis is 25 times greater than that for the rat SERT *y*-axis, consistent with the differences in the potency of imipramine for the two SERTs.

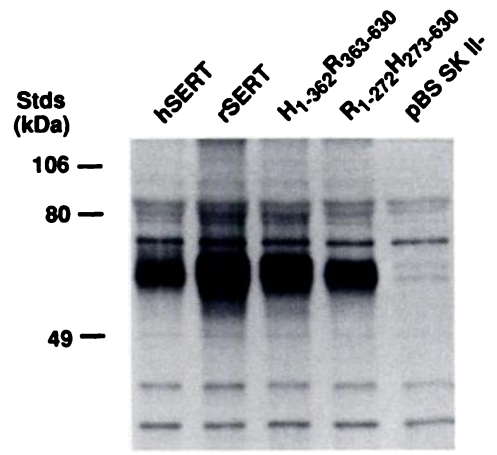

Fig. 2. Immunoprecipitation of parental and chimeric SERTs. [^{35}S]Methionine/cysteine-labeled HeLa cells that had been transfected with either parental or chimeric SERTs were subjected to immunoprecipitation with the S365 antibody as described in Materials and Methods. Transfection with the plasmid pBluescript SK II(−) (*pBS SK II−*) lacking SERT cDNA was used as a control. *Numbers at the left*, relative molecular mass standards. Chimera $H_{1-362}R_{363-631}H_{632-630}$ also was immunoprecipitated, with a molecular mass similar to that of parental SERTs (not shown). *hSERT*, human SERT; *rSERT*, rat SERT.

SERT, compared with the human homolog. Heterocyclic antagonists such as fluoxetine, cocaine, and paroxetine displayed no discernible species selectivity, nor did the substrate 5-HT appear to be transported with any differences in kinetic parameters. The differential affinity for human SERT displayed by imipramine was directly demonstrated by radioligand binding using [^{3}H]imipramine. Furthermore, the increase in potency associated with imipramine binding was shown not to arise from species differences in the ion dependence of imipramine binding, because rat and human SERTs exhibited similar ion-dependence profiles for imipramine affinity. Recently we have also directly compared the cloned human SERT with the cloned *Drosophila* SERT in transfected cells, revealing marked shifts in the potencies of SERT inhibitors for the *Drosophila* transporter despite equivalent recognition of the preferred substrate 5-HT (6).

Our findings provide a molecular basis for differential pharmacological profiles obtained with endogenous SERTs compared across species. Wielosz *et al.* (10) were the first to show that the tricyclic antidepressants imipramine and clomipramine were more potent at blocking 5-HT uptake in human platelets, compared with rat platelets. Desipramine and fluoxetine also have been described as being more potent inhibitors of 5-HT uptake in human versus rat brain slices (19), whereas citalopram appears to have higher affinity at the rat SERT in binding assays using rat and human membrane preparations (18). In contrast to these findings showing species selectivity for fluoxetine and citalopram, we found no such species preferences for these drugs when they were compared using the cloned SERTs. Several tricyclic antidepressant compounds also have been reported to display greater potencies at the rat transporter, compared with the guinea pig transporter (20), although cloned guinea pig SERTs are not presently available for direct comparison in transfected cells. Intrinsic differences in peripheral and brain SERTs could explain some of these observed variations in pharmacological sensitivity. Although cell-specific post-translational modifications are evident for endogenous SERTs (24), a single gene appears to encode human and rodent SERTs expressed in both the brain and the periphery (3) and, furthermore, we have confirmed species-selective pharmacologies using a single-cell type expression system. We conclude that the observed species differences reflect inherent variations in the potencies of antagonists for the two mammalian SERT homologs, resulting from amino acid sequence variations. Our results also suggest caution in extrapolation of *in vivo* pharmacological data on 5-HT uptake inhibitors from rodents to humans, especially in drug development, where absolute potency or rank order of potency is often important. For example, the rank order of potency for some antagonists changes depending upon which species homolog is examined (e.g., human SERT, clomipramine < citalopram = imipramine

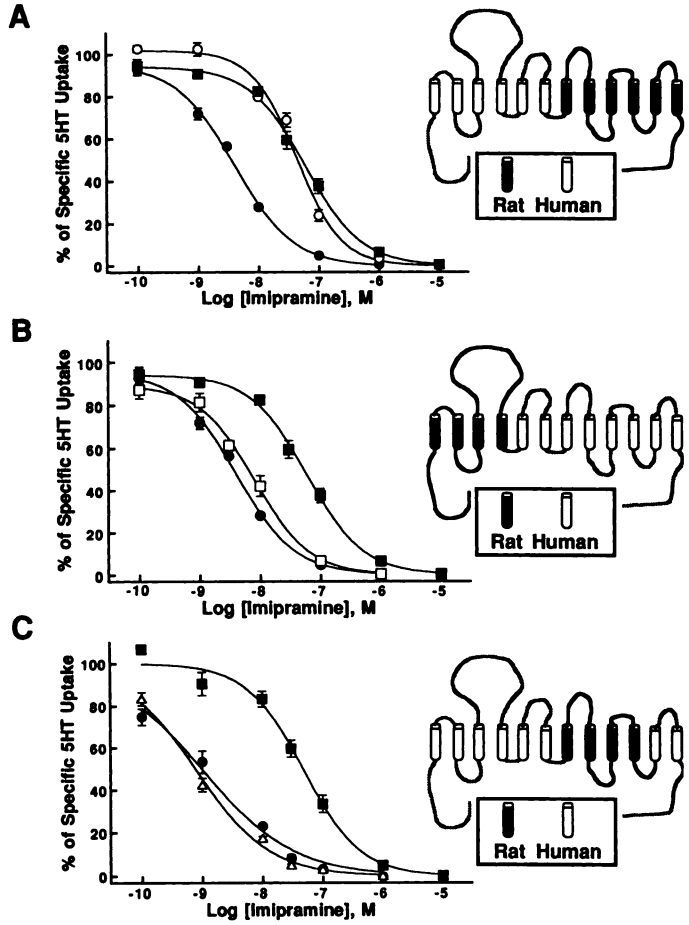

Fig. 3. Imipramine inhibition of 5-HT uptake at parental and chimeric SERTs. [^{3}H]5-HT uptake assays were performed on transiently transfected HeLa cells, as described in Materials and Methods, with increasing concentrations of imipramine added 10 min before the addition of 20 nM [^{3}H]5-HT. Mean nonspecific uptake determined in HeLa cells transfected with the parent vector pBluescript SK II(−) was 68 ± 3 cpm. A, Evaluation of imipramine potency for human SERT (●), rat SERT (■), and chimera $H_{1-362}R_{363-630}$ (○). Data were plotted as percentage of specific 5-HT uptake (human SERT, 4630 ± 80 cpm; rat SERT, 6860 ± 610 cpm; chimera $H_{1-362}R_{363-630}$, 5230 ± 90 cpm). Mean K_i values are presented in Table 1. B, Evaluation of imipramine potency for human SERT (●), rat SERT (■), and chimera $R_{1-272}H_{273-630}$ (□). Data were plotted as percentage of specific 5-HT uptake (human SERT, 4630 ± 80 cpm; rat SERT, 6860 ± 610 cpm; chimera $R_{1-272}H_{273-630}$, 2120 ± 120 cpm). Mean K_i values are presented in Table 1. C, Evaluation of imipramine potency for human SERT (●), rat SERT (■), and chimera $H_{1-362}R_{363-531}H_{532-630}$ (△). Data were plotted as percentage of specific 5-HT uptake (human SERT, 1390 ± 40 cpm; rat SERT, 2160 ± 50 cpm; chimera $H_{1-362}R_{363-531}H_{532-630}$, 1010 ± 30 cpm). Mean K_i values are as follows: human SERT, 1.2 ± 0.3 nM; rat SERT, 47 ± 10 nM; chimera $H_{1-362}R_{363-531}H_{532-630}$, 7.6 ± 1.3 nM. All data plotted represent means ± standard errors of triplicate determinations and are representative of three (A and B) or two (C) separate experiments.

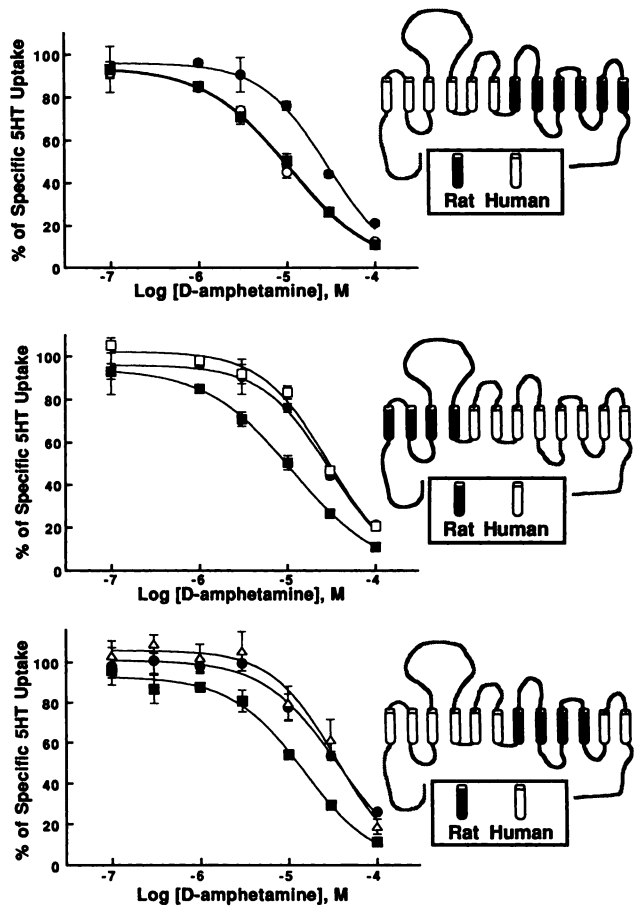

Fig. 4. *d*-Amphetamine inhibition of 5-HT uptake at parental and chimeric SERTs. [^{3}H]5-HT uptake assays were performed in transiently transfected HeLa cells, as described in Materials and Methods, with increasing concentrations of *d*-amphetamine added simultaneously with the addition of 20 nM [^{3}H]5-HT. Mean nonspecific uptake determined in HeLa cells transfected with the parent vector pBluescript SK II(−) was 49 ± 3 cpm. A, Evaluation of *d*-amphetamine potency for human SERT (●), rat SERT (■), and chimera $H_{1-362}R_{363-630}$ (○). Data were plotted as percentage of specific 5-HT uptake (human SERT, 1970 ± 180 cpm; rat SERT, 4890 ± 160 cpm; chimera $H_{1-362}R_{363-630}$, 3490 ± 70 cpm). Mean K_i values are presented in Table 1. B, Evaluation of *d*-amphetamine potency for human SERT (●), rat SERT (■), and chimera $R_{1-272}H_{273-630}$ (□). Data were plotted as percentage of specific 5-HT uptake (human SERT, 1970 ± 180 cpm; rat SERT, 4890 ± 160 cpm; chimera $R_{1-272}H_{273-630}$, 780 ± 60 cpm). Mean K_i values are presented in Table 1. C, Evaluation of *d*-amphetamine potency for human SERT (●), rat SERT (■), and chimera $H_{1-362}R_{363-531}H_{532-630}$ (△). Data were plotted as percentage of specific 5-HT uptake (human SERT, 890 ± 60 cpm; rat SERT, 1380 ± 30 cpm; chimera $H_{1-362}R_{363-531}H_{532-630}$, 910 ± 50 cpm). Mean K_i values are as follows: human SERT, 35 ± 3.5 μM; rat SERT, 14 ± 1.6 μM; chimera $H_{1-362}R_{363-531}H_{532-630}$, 33 ± 4.9 μM. All data plotted represent means ± standard errors of triplicate determinations and are representative of three (A and B) or two (C) separate experiments.

< quipazine; rat SERT, clomipramine = citalopram < quipazine < imipramine). Heterologously expressed human SERTs are likely to be a more appropriate model for human drug design than are rodent-based screens for SERT antagonists.

Functional expression of SERT cDNAs in mammalian cells provides strong evidence that a single protein is capable of both eliciting 5-HT uptake and serving as a target for SERT inhibitors. In an examination of the protective capacity of different antagonists against the alkylating agent *N*-ethylmaleimide, Graham *et al.* (9) obtained data for common and/or overlapping binding sites for the tricyclic and nontricyclic SERT antagonists at the 5-HT recognition site. Our data are consistent with this hypothesis but indicate that some variations exist in the molecular interactions of different drugs with the transporter. In this regard, biochemical evidence has been generated indicating that SERT antagonists have distinct binding domains on SERT proteins. Chemical inactivation studies with *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline suggest that the tricyclic and nontricyclic SERT antagonists have at least two nonoverlapping binding sites (27). Radioligand binding data examining the kinetics of [^{3}H]imipramine dissociation in

the presence of various antagonists also suggest heterogeneity in the binding of SERT antagonists (28). The simplest explanation for species differences for some, but not all, SERT antagonists is the existence of distinct transporter-ligand contact sites that are unique to the different chemical classes of inhibitors. Chimera $H_{1-362}R_{363-630}$ consistently exhibited enhanced potency for cocaine, relative to either parental SERT, suggesting that the cocaine binding site may include regions both proximal and distal to the chimera junction. Because cocaine exhibits no species selectivity, alternative strategies are required to localize specific cocaine contact sites (29).

In addition to confirming that antagonists bind to SERTs in distinct manners, our data indicate that SERT substrate recognition sites may vary as well. Whereas the indoleamine 5-HT showed no K_m differences between rat and human SERTs, *d*-amphetamine, a phenethylamine, showed species preferences opposite to those described for the tricyclic antidepressants. Chemical modification of the amphetamine nucleus, as in *p*-chloroamphetamine and MDMA, appears to alter the protein-drug contact sites such that no consistent and localizable species preferences were observed. Our findings are consistent with those of Wolf and Kuhn (30), who demonstrated that 5-HT can protect SERTs from inactivation with alkylating agents but that amphetamines do not protect SERTs. If the different SERT substrates recruit distinct binding domains, as suggested, then the use of alternative substrates may be informative in evaluations of mutation-induced alterations in transporter function where recognition of one substrate is lost but recognition of other substrates may be retained. If multiple binding sites distinct from substrate recognition domains exist on SERTs, then drugs targeted to these ligand subsites might be capable of blocking the interactions of some inhibitors, such as cocaine or the neurotoxic amphetamines, at SERTs without altering native 5-HT uptake activity.

Our finding that nortriptyline was the only tricyclic antidepressant examined that did not exhibit significant species selectivity implies that subtle changes in structure within chemical classes of inhibitors may alter the ability of the compounds to interact with proteins and may thus reveal important information about the pharmacophore of the compounds. It is well established that the tertiary amine compounds (imipramine and amitriptyline) are significantly more selective for SERT than are the secondary amines (desipramine, nortriptyline, and protriptyline), which are more potent at NET (1, 31). This implicates the functional groups on the amino moiety in mediating transporter selectivity of these compounds. Our results also suggest that the orientation of the amino group subtituents is important for the species selectivity of the tricyclic antagonists. Desipramine, protriptyline, and nortriptyline are all secondary amines (Fig. 5A), but only nortriptyline fails to exhibit species selectivity. Of these drugs, nortriptyline is the only one possessing a double bond connecting the amine-containing alkyl chain to the center ring of the tricyclic nucleus; this constrains the alkyl chain to a planar conformation. Amitriptyline, which displays species selectivity, has a planar conformation in the linkage of the alkyl chain similar to that of nortriptyline but is a tertiary amine. Therefore, the combination of the secondary amine in the planar conformation results in the loss of species selectivity. We propose that the spatial locations of the terminal amine and its methyl groups are

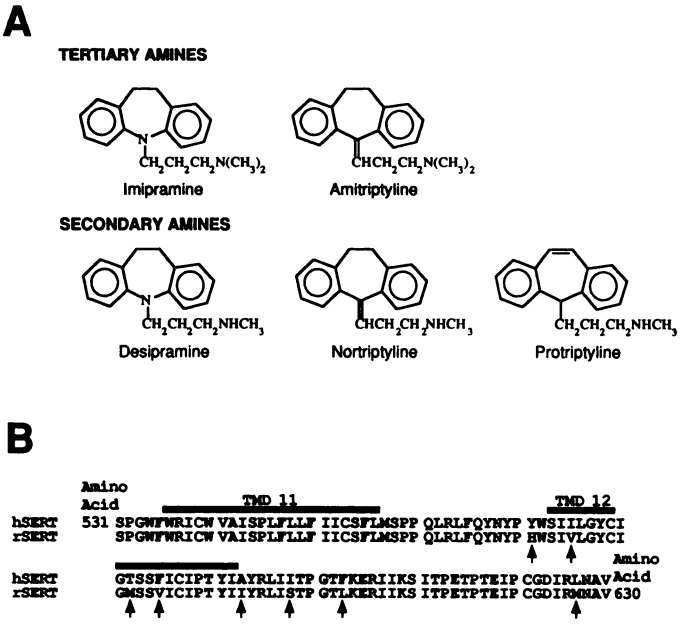

Fig. 5. A, Comparison of tricyclic antidepressant structures. B, Comparison of amino acids 531–630 from human SERT (*hSERT*) and rat SERT (*rSERT*) presumed to be involved in high affinity interactions of species-selective SERT ligands. *Black bars*, TMDs 11 and 12; *arrows*, points of sequence divergence. Rat SERT and human SERT share 92% overall sequence identity.

involved in the chemical basis of SERT antidepressant species selectivity.

Species differences in antagonist potency most likely arise from the limited number of amino acid sequence differences between rat and human SERTs. SERT chimera analysis has allowed us to localize the domain dictating species-specific drug selectivity. We found that cross-species chimeras between the rat and human SERTs were synthesized similarly by the HeLa cells, as evaluated by immunoprecipitations, and produced functional transporters that transported 5-HT with K_m and V_{max} values comparable to those of parental SERTs. This is in contrast to our previous attempts to obtain functional chimeras between the more divergent NET and SERT, where many chimeras were found to be inactive (32). When the rat/human SERT chimera contained human TMDs 1–6 and rat TMDs 7–12, as in chimera $H_{1-362}R_{363-630}$, the pharmacological profile was similar to that of rat SERT, whereas the $R_{1-272}H_{273-630}$ chimera, which possessed human TMDs 5–12, appeared more like human SERT (Figs. 3 and 4; Table 1). Taken together, these data suggested that the higher affinity for tricyclic compounds and the lower affinity for amphetamine observed for human SERT are mediated by interactions distal to amino acid 363. Examination of chimera $H_{1-362}R_{363-531}H_{532-630}$ further localized the domains involved in species-selective pharmacologies of rat and human SERTs to amino acids 532–630, which include TMDs 11 and 12 and the carboxyl terminus.

After localization of the region of SERT involved in species-selective drug potencies by using the cross-species chimeras, direct comparison of the amino acid sequences of the rat and human SERTs revealed only eight amino acid switches in the area restricted to amino acids 532–630 (Fig. 5B). Switching of the carboxyl tails of SERT and NET has previously been shown to preserve antagonist selectivity and potencies, suggesting that the carboxyl terminus distal to TMD 12 is not involved in the

ligand recognition differences reported here (33). As in β-adrenergic receptors (34), the ligand binding pocket of SERT may be composed of the hydrophobic TMD regions. Within SERT TMDs 11 and 12, there are only five switches between rat and human SERTs, all of which are located in TMD 12. Additional support for the TMD location of transporter antagonist contact sites comes from additional studies with SERT as well as other monoamine transporters, where mutations of residues within TMDs 1, 7, and 11 detrimentally affect the binding of substrates and antagonists (29, 35, 36). The power of a single amino acid substitution to alter antagonist potencies across species has been amply demonstrated for many G protein-coupled receptors and ion channels. For example, studies with the human 5-$HT_{1D\beta}$ receptor (previously known as the 5-HT_{1B} receptor) reveal that the mutation of a single threonine residue to its counterpart in the rat receptor, an asparagine, results in the human receptor taking on pharmacological characteristics almost identical to those of the rat receptor (16). Although it is premature to speculate on the precise sites of antagonist binding to SERTs, substrates like *d*-amphetamine may be required to interact within TMDs if translocation into the cytoplasm is to occur. Our localization of modulatory contact sites for substrates and tricyclic antidepressants is consistent with the findings from NET/dopamine transporter chimera studies of Giros *et al.* (37). Those investigators attributed *d*-amphetamine recognition, in part, to regions of homologous catecholamine transporters distal to TMD 9. Furthermore, major determinants for tricyclic antidepressant recognition by NET were localized to TMDs 6–8 and intervening loops. In the context of our findings, these data suggest that tricyclic antidepressant binding may be mediated by both essential and modulatory contact sites in the distal halves of NETs and SERTs. Future studies exploiting comparisons of cross-species chimeras coupled with rational site-directed mutagenesis of SERTs and NETs should be able to pinpoint residues involved in the binding of transporter ligands.

References

1. Blakely, R. D., H. E. Berson, R. T. J. Fremeau, M. G. Caron, M. M. Peek, H. K. Prince, and C. C. Bradley. Cloning and expression of a functional serotonin transporter from rat brain. *Nature (Lond.)* **354**:66–70 (1991).
2. Hoffman, B. J., E. Mezey, and M. J. Brownstein. Cloning of a serotonin transporter affected by antidepressants. *Science (Washington D. C.)* **254**:579–580 (1991).
3. Ramamoorthy, S., A. L. Bauman, K. R. Moore, H. Han, T. Yang Feng, A. S. Chang, V. Ganapathy, and R. D. Blakely. Antidepressant- and cocaine-sensitive human serotonin transporter: molecular cloning, expression, and chromosomal localization. *Proc. Natl. Acad. Sci. USA* **90**:2542–2546 (1993).
4. Chang, A. S., S. M. Chang, D. M. Starnes, and R. D. Blakely. Cloning and expression of the mouse brain serotonin transporter. *Soc. Neurosci. Abstr.* **19**:495 (1993).
5. Corey, J. L., M. W. Quick, N. Davidson, H. A. Lester, and J. Guastella. A cocaine-sensitive *Drosophila* serotonin transporter: cloning, expression, and electrophysiological characterization. *Proc. Natl. Acad. Sci. USA* **91**:1188–1192 (1994).
6. Demchyshyn, L. L., Z. B. Pristupa, K. S. Sugamori, E. L. Barker, R. D. Blakely, W. J. Wolfgang, M. A. Forte, and H. B. Niznik. Cloning, expression, and localization of a chloride-facilitated cocaine-sensitive serotonin transporter from *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **91**:5158–5162 (1994).
7. Amara, S. G., and M. J. Kuhar. Neurotransmitter transporters: recent progress. *Annu. Rev. Neurosci.* **16**:73–93 (1993).
8. Marcusson, J. O., and S. B. Ross. Binding of some antidepressants to the 5-hydroxytryptamine transporter in brain and platelets. *Psychopharmacology (Berl.)* **102**:145–155 (1990).
9. Graham, D., H. Esnaud, E. Habert, and S. Z. Langer. A common binding site for tricyclic and nontricyclic 5-hydroxytryptamine uptake inhibitors at the substrate recognition site of the neuronal sodium-dependent 5-hydroxytryptamine transporter. *Biochem. Pharmacol.* **38**:3819–3826 (1989).
10. Wielosz, M., M. Salmona, G. de Gaetano, and S. Garattini. Uptake of ^{14}C-5-hydroxytryptamine by human and rat platelets and its pharmacological inhibition: a comparative kinetic analysis. *Naunyn-Schmiedebergs Arch. Pharmacol.* **29**:59–65 (1976).
11. Cool, D. R., F. H. Leibach, and V. Ganapathy. High affinity paroxetine binding to the human placental serotonin transporter. *Am. J. Physiol.* **259**:C196–C204 (1990).
12. Humphreys, C. J., D. Beidler, and G. Rudnick. Substrate and inhibitor binding and translocation by the platelet plasma membrane serotonin transporter. *Biochem. Soc. Trans.* **19**:95–98 (1991).
13. Humphreys, C. J., J. Levin, and G. Rudnick. Antidepressant binding to the porcine and human platelet serotonin transporters. *Mol. Pharmacol.* **33**:657–663 (1988).
14. Rudnick, G., J. Talvenheimo, H. Fishkes, and P. J. Nelson. Sodium ion requirements for serotonin transport and imipramine binding. *Psychopharmacol. Bull.* **19**:545–549 (1983).
15. Hall, J. M., M. P. Caulfield, S. P. Watson, and S. Guard. Receptor subtypes or species homologues: relevance to drug discovery. *Trends Pharmacol. Sci.* **14**:376–383 (1993).
16. Oksenberg, D., S. A. Marsters, B. F. O'Dowd, H. Jin, S. Havlik, S. J. Peroutka, and A. Ashkenazi. A single amino-acid difference confers major pharmacological variation between human and rodent 5-HT_{1B} receptors. *Nature (Lond.)* **360**:161–163 (1992).
17. Fong, T. M., H. Yu, and C. D. Strader. Molecular basis for the species selectivity of the neurokinin-1 receptor antagonists CP-96,345 and RP67580. *J. Biol. Chem.* **267**:25668–25671 (1992).
18. Plenge, P., and E. T. Mellerup. [^{3}H]Citalopram binding to brain and platelet membranes of human and rat. *J. Neurochem.* **56**:248–252 (1991).
19. Harms, H. H. The antidepressant agents desipramine, fluoxetine, fluvoxamine and norzimelidine inhibit uptake of noradrenaline and 5-hydroxytryptamine in slices of human and rat cortical brain tissue. *Brain Res.* **275**:99–104 (1983).
20. Hornsby, C. D., J. M. Barnes, N. M. Barnes, S. Champaneria, B. Costall, and R. J. Naylor. Pharmacological comparison of the rat and guinea-pig cortical high affinity 5-hydroxytryptamine uptake system. *Biochem. Pharmacol.* **43**:1865–1868 (1992).
21. Blakely, R. D., J. A. Clark, G. Rudnick, and S. G. Amara. Vaccinia-T7 RNA polymerase expression system: evaluation for the expression cloning of plasma membrane transporters. *Anal. Biochem.* **194**:302–308 (1991).
22. Fuerst, T. R., E. Niles, F. W. Studier, and B. Moss. Eukaryotic transient expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* **83**:8122–8126 (1986).
23. Cheng, Y., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of an inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
24. Qian, Y., H. E. Melikian, D. B. Rye, A. I. Levey, and R. D. Blakely. Identification and characterization of antidepressant-sensitive serotonin transporter proteins. *J. Neurosci.*, in press.
25. Rudnick, G., and S. C. Wall. *p*-Chloroamphetamine induces serotonin release through serotonin transporters. *Biochemistry* **31**:6710–6718 (1992).
26. Rudnick, G., and S. C. Wall. The molecular mechanism of "ecstasy": serotonin transporters are targets for MDMA-induced serotonin release. *Proc. Natl. Acad. Sci. USA* **89**:1817–1821 (1992).
27. Biessen, E. A., J. A. Norder, A. S. Horn, and G. T. Robillard. Evidence for the existence of at least two different binding sites for 5-HT-reuptake inhibitors within the 5-HT-reuptake system from human platelets. *Biochem. Pharmacol.* **37**:3959–3966 (1988).
28. Plenge, P., E. T. Mellerup, T. Honore, and P. L. Honore. The activity of 25 paroxetine/femoxetine structure variants in various reactions, assumed to be important for the effect of antidepressants. *J. Pharm. Pharmacol.* **39**:877–882 (1987).
29. Kitayama, S., S. Shimada, H. Xu, L. Markham, D. M. Donovan, and G. R. Uhl. Dopamine transporter site-directed mutations differentially alter substrate transport and cocaine binding. *Proc. Natl. Acad. Sci. USA* **89**:7782–7785 (1992).
30. Wolf, W. A., and D. M. Kuhn. Role of essential sulfhydryl groups in drug interactions at the neuronal 5-HT transporter: differences between amphetamines and 5-HT uptake inhibitors. *J. Biol. Chem.* **267**:20820–20825 (1992).
31. Pacholczyk, T., R. D. Blakely, and S. G. Amara. Expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter. *Nature (Lond.)* **350**:350–354 (1991).
32. Moore, K. R., and R. D. Blakely. Restriction site-independent formation of chimeras from homologous neurotransmitter transporter cDNAs. *Biotechniques* **17**:130–136 (1994).

33. Blakely, R. D., K. R. Moore, and Y. Qian. Tails of serotonin and norepinephrine transporters: deletions and chimeras retain function. *Soc. Gen. Physiol. Ser.* **48**:283–300 (1993).
34. Kobilka, B. Adrenergic receptors as models for G protein-coupled receptors. *Annu. Rev. Neurosci.* **15**:87–114 (1992).
35. Melikian, H. E., K. R. Moore, Y. Qian, H. L. Kimmel, S. B. Taylor, R. W. Gereau, A. Levey, and R. D. Blakely. Structure and function of plasma membrane serotonin transporters. *Soc. Neurosci. Abstr.* **19**:494 (1993).
36. Kitayama, S., J. B. Wang, and G. R. Uhl. Dopamine transporter mutants selectively enhance MPP^+ transport. *Synapse* **15**:58–62 (1993).
37. Giros, B., Y. M. Wang, S. Suter, S. B. McLeskey, C. Pifl, and M. G. Caron. Delineation of discrete domains for substrate, cocaine, and tricyclic antidepressant interactions using chimeric dopamine-norepinephrine transporters. *J. Biol. Chem.* **269**:15985–15988 (1994).

Send reprint requests to: Randy D. Blakely, Department of Anatomy and Cell Biology, Emory University School of Medicine, Atlanta, GA 30322.