DISTINCT RECOGNITION OF SUBSTRATES BY THE HUMAN AND DROSOPHILA SEROTONIN TRANSPORTERS.


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Abstract

The human and Drosophila serotonin transporters (hSERT and dSERT, respectively) were used to explore differences in substrate properties. hSERT and dSERT showed similar \( K_m \) values for 5-HT transport (1.2 and 0.9 \( \mu \)M, respectively) suggesting similar recognition of 5-HT by the two species variants. Although dSERT cell surface expression was approximately eight-fold lower than hSERT, dSERT does appear to have a two-fold faster turnover number for inward transport of 5-HT. Interestingly, another substrate N-methyl-4-phenylpyridinium (MPP\(^+\)) was transported only by hSERT. However, MPP\(^+\) inhibited 5-HT uptake in both species variants with similar potencies. Two cross-species chimeras, \( \text{H}^{1-118}\text{D}^{119-627} \) and \( \text{H}^{1-281}\text{D}^{282-476}\text{H}^{477-638} \), were also unable to transport MPP\(^+\) implicating the role of TMDs V to IX in the substrate permeation pathway. Based on exchange experiments, certain substituted-amphetamines also appear to be poor substrates at dSERT. Two-electrode voltage-clamp studies in oocytes confirmed that the amphetamines do not possess substrate-like properties for dSERT. Our data suggest distinct molecular recognition among SERT substrate classes that influence translocation mechanisms.
Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine neurotransmitter that plays an essential role in the nervous system. 5-HT is important to physiological processes including smooth muscle tone, memory, appetite, and mood (Jacobs et al., 1995). 5-HT signaling is regulated by a diverse set of mechanisms, including biosynthetic enzymes, secretory proteins, ion channels, pre- and postsynaptic receptors, and transporters. The synaptic concentration of 5-HT is regulated by sodium- and chloride-dependent serotonin transporters (SERTs; Barker and Blakely, 1995) that couple uptake to an electrochemical gradient, thus, facilitating inward translocation of the neurotransmitter (Gu et al., 1994; Rudnick, 1998). Besides a role in the termination of synaptic 5-HT signals, SERT is the target of various clinical drugs such as tricyclic antidepressants (TCAs; imipramine, amitriptyline) and selective serotonin reuptake inhibitors (SSRIs; fluoxetine, paroxetine, citalopram). Abused drugs such as cocaine and psychoactive amphetamines also inhibit the 5-HT transport mechanism. SERT antagonists including the TCAs, SSRIs, and cocaine are thought to inhibit the translocation process by binding to the transporter (Barker and Blakely, 1995; White, 1998). The amphetamines (3,4-methylenedioxyamphetamine (MDMA), p-chloroamphetamine), like 5-HT itself, are substrates for SERT and induce an outward movement or exchange of 5-HT from the cytoplasm through SERTs (Rudnick and Wall, 1992a, b). Although the molecular mechanism by which the amphetamines promote non-vesicular 5-HT release is not well understood, previous studies suggest that the inward movement of amphetamine leads to reversal of the transport process and results in the net exchange of intracellular 5-HT (Rudnick and Wall, 1993; Wall et al., 1995).
Cloning of SERT from several organisms including rat (Blakely et al., 1991; Hoffman et al., 1991), human (Ramamoorthy et al., 1993), and Drosophila (Demchyshyn et al., 1994; Corey et al., 1994) revealed shared sequence identity with other members of the sodium- and chloride-dependent γ-aminobutyric acid (GABA)/norepinephrine (GAT/NET) transporter gene family. Similar to other transporter homologues, sequence analysis led to the prediction that SERTs possess twelve transmembrane domains (TMDs) with both amino and carboxyl terminals localized to the cytoplasm. SERT sequence analysis also predicts a large extracellular loop between TMD III and TMD IV containing multiple N-linked glycosylation sites and several putative intracellular domains for phosphorylation by protein kinases. By using various site-specific labeling techniques, the topology of this gene family has been largely confirmed (Bruss et al., 1995; Chen et al., 1998; Ferrer and Javitch, 1998; Androutsellis-Theotokis and Rudnick, 2002). Despite the sequence homology and similar $K_m$ values for 5-HT transport between SERT species variants, several lines of evidence demonstrate species differences in antagonist recognition (Barker et al., 1994; Barker et al., 1998) and the recognition of tryptamine analogs (Adkins et al., 2001). These studies have revealed that single amino acid substitutions across SERT species variants are sufficient to alter ligand recognition. Amino acids involved with species-specific pharmacologic properties of SERT may play a role in maintaining a favorable conformation for ligand recognition or may directly participate in ligand binding as part of the drug binding pocket.

Little information is available on the molecular determinants of substrate recognition and translocation by SERT. In the present study, we investigated
differences in the properties of SERT substrates between the human and *Drosophila* SERTs (hSERT and dSERT, respectively). hSERT (630 amino acids) and dSERT (622 amino acids) share 51% sequence identity and demonstrate similar $K_m$ values for 5-HT uptake (Demchyshyn et al., 1994). We speculated that hSERT and dSERT might demonstrate marked differences for the transport of other substrates such as N-methyl-4-phenylpyridinium (MPP$^+$) and the amphetamines, providing opportunities to exploit these species-specific properties in molecular studies to reveal structural information about substrate recognition and permeation. Indeed, hSERT readily transported the neurotoxic compound MPP$^+$, however, MPP$^+$ was not transported by dSERT. Moreover, amphetamine analogs were not readily transported by dSERT as determined by 5-HT release assays and electrophysiology experiments. Finally, cross-species chimeras between hSERT and dSERT were used to implicate the region from TMD V to IX of SERT as containing structural components involved with substrate recognition.
Materials and Methods

Materials

HEK-293 cell lines stably expressing hSERT or dSERT were generous gifts from Dr. Randy D. Blakely (Vanderbilt University). [3H] 5-hydroxytryptamine ([3H] 5-HT; 122 Ci/mmol) and [3H] citalopram (85 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). [3H] Mazindol (21 Ci/mmol) and [3H] MPP+ (78 Ci/mmol) were obtained from NEN Life Science Products (Boston, MA). Fluoxetine, MPP+, and pargyline were purchased from Research Biochemical International (Natick, MA). Unlabeled 5-HT was from Sigma (St. Louis, MO). Amphetamines were synthesized using conventional methods. Their characterization was carried out using NMR, MS, and elemental analysis and all data were consistent with the expected structures. All other reagents were purchased from commercial sources.

Chimera construction

The generation of the H1-118D119-627 cross-species chimera between hSERT and dSERT using a restriction site-independent method of chimera formation was previously described (Moore and Blakely, 1994; Barker et al., 1998). The resulting cross-species chimera encoded amino acids 1-118 from hSERT and 119-627 from dSERT. Construction of the H1-281D282-476H477-638 chimera was initiated using the Quick-Change mutagenesis kit (Strategene, La Jolla, CA) to make a silent mutation in the Drosophila SERT cDNA that introduced a BsiWI restriction site at position 1419 from the first position of the coding region. The same method was used on the human SERT cDNA to introduce a complementary BsiWI site (from position 1446 of the initiating codon), as well as an EcoNI site at position 808 from the first position of the coding region. A
complementary EcoNI restriction site is native in dSERT (Fig 1A). Mutations were confirmed by restriction enzyme digestion and nucleotide sequencing. Each SERT cDNA was digested with EcoNI and BsiWI and the resulting fragments were gel-purified and complementary fragments were ligated to yield the chimeric cDNA. The resulting construct encoded amino acids 1-281 from hSERT, 282-476 from dSERT, and hSERT from 477-638 (Fig. 1A).

HEK-293 cells stably expressing the hSERT/dSERT chimeras were produced as described elsewhere (Qian et al., 1997). Briefly, chimeric cDNAs in pBluescript KSII* were digested with XhoI/XbaI and subcloned into pcDNA 3.1 (Invitrogen, San Diego, CA). Wild type HEK-293 cells were transfected with either H1-118D119-627/pcDNA 3.1 or H1-281D282-476H477-638 /pcDNA 3.1 using a lipid-mediated transfer (Lipofectamine 2000; Life Technologies, Gaithersburg, MD), as described by the manufacturer. Transfected cells were selected with 600 mg/L geneticin (G418; Gibco BRL, Grand Island, NY). Selected colonies were characterized for [3H] 5-HT uptake.

HEK-293 cells stably expressing hSERT, dSERT, or chimeras were maintained in Dulbecco’s modified Eagle medium (DMEM) with 10% dialyzed fetal bovine serum supplemented with penicillin, streptomycin, L-glutamine, and G-418 (600 mg/L). Cells were grown in a 37 °C humidified environment with 5% CO2.

[3H] Substrate uptake assays

Saturation transport assays were performed in 24-well culture plates precoated with poly-D-Lysine. At the time of assay, cells (1 x 10^5 cells per well) were washed 1x with Krebs/Ringer/Hepes (KRH) buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl$_2$, 10 mM Heps, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, pH 7.4). Cells were incubated with
increasing concentrations of substrate for 10 minutes at 37 °C in KRH containing D-glucose (1.8 g/L), L-ascorbic acid (100 µM), and pargyline (100 µM). Fluoxetine (10 µM) was used for nonspecific uptake. Saturation kinetics were determined using increasing concentrations of [³H] 5-HT (0.625 - 20 µM) or [³H] MPP⁺ (12.5 – 400 µM) with a specific activity diluted to ~0.1 Ci/mmol with unlabeled compound. Assays were terminated by washing three times with KRH buffer. The amount of remaining radiolabeled substrate was then determined using a Packard TopCount-NXT Microplate Scintillation and Luminescence Counter (Meriden, CT). Saturation assays on the substituted-amphetamines were not performed because radiolabeled compounds were not available, and furthermore, these compounds are extremely lipophilic, thus, preventing accurate determination of the transport kinetics.

For uptake inhibition assays, cells were plated as described for release assays. At the time of assay, the plates were washed with KRH buffer, incubated with increasing concentration of drug at 37 °C for 10 minutes, then treated with [³H] 5-HT (20 nM). Cells were incubated another 10 minutes and uptake was terminated by harvesting into a 96-well GF/B filter plate. Accumulated [³H] 5-HT was determined as described above.

[³H] 5-HT exchange assays

For release assays, cells were plated in tissue culture dishes (150 mm x 20 mm) and incubated at 37 °C for 2 days (approximately 90% confluent). Cells were resuspended in KRH/D-glucose (15 mL final volume). Protein concentrations were determined using the Bradford method (Bio-Rad, Hercules, CA) and suspensions diluted to 0.5 mg/mL with KRH/D-glucose buffer. At the time of assay, 50 µg of protein (100 µL) was incubated with 100 µL of [³H] 5-HT (50 nM final concentration) at 37 °C for
five min (hSERT) or thirty min (dSERT, H¹-¹¹⁸D¹¹⁹⁻⁶²⁷, and H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸) to provide for equivalent loading. The cell suspensions were treated with increasing concentrations of drug in a final volume of 1 mL. Time-course experiments (not shown) revealed that exchange at both hSERT and dSERT reached maximum at 10 min. Thus, cells were incubated for ten minutes, and then exchange was terminated by harvesting into a 96-well GF/B filter plate. The amount of remaining radiolabeled substrate was determined using liquid scintillation spectrometry as described above.

**Whole-cell radioligand binding assay**

Whole-cell binding experiments were performed in six-well plates (1x10⁶ cells per well) precoated with poly-D-Lysine. The day after plating, cells were washed 1x with KRH. A saturating concentration (20 nM) of [³H] citalopram or [³H] mazindol was used as a radiolabeled ligand for hSERT and dSERT, respectively. Radiolabeled ligand and cells were incubated at 4 °C for 1 hour. After incubation, cells were rapidly washed 2x with 1000 µL KRH, solubilized with 10% SDS solution, and transferred into a scintillation vial. [³H] ligand was determined using a Beckman LS 1801 liquid scintillation counter (Irvine, CA). Total binding was established in the presence of KRH buffer. Nonspecific binding was defined as the binding of radiolabeled ligand in the presence of fluoxetine (10 µM). Internal binding was determined in the presence of MPP⁺ (400 µM).

**Two-electrode voltage-clamp of SERT-expressing Xenopus oocytes**

hSERT and dSERT cDNAs were subcloned into the *Xenopus* transcription vector, OTV (gift of Dr. Susan Amara, Vollum Institute, Oregon Health & Science University). H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸/OTV was constructed by subcloning D²⁸²⁻⁴⁷⁶ into hSERT/OTV using the restriction sites described in this paper. Construction was
confirmed enzymatically with BglII and AflIII. All cDNAs were linearized with XbaI. hSERT, dSERT, and \(^{1-281}D^{282-476}H^{477-638}\) cRNA was transcribed using the T7 mMessage mMachine \textit{In Vitro} Transcription Kit (Ambion, Inc., Austin, TX).

Defolliculated stage V and VI oocytes were injected with 45-60 ng of either hSERT, dSERT, or \(^{1-281}D^{282-476}H^{477-638}\) cRNA and maintained in Ca\(^{2+}\)-Ringer’s solution (2 mM KCl, 5mM MgCl\(_2\), 96 mM NaCl, 5 mM HEPES, and 0.6 mM CaCl\(_2\), pH 7.6) supplemented with 5% horse serum (Sigma) and 1% penicillin/streptomycin (Life Technologies/GIBCO) at 18 °C. Recordings were performed 6-7 d after cRNA injection. Oocytes used in recording experiments exhibited resting membrane potentials ranging from -25 to -63 mV.

SERT-expressing oocytes were subjected to two-electrode voltage-clamp using an AxoClamp 2B (Axon Instruments, Foster City, CA). Glass microelectrodes were pulled to a resistance of 0.2-1.5 M\(\Omega\) and filled with 3M KCl. Recording solutions consisted of room temperature Ca\(^{2+}\)-Ringer’s solution with 5-HT, fluoxetine, or 5-methoxy-6-methyl-2-aminindan (MMAI) as indicated. Transporter-associated currents were recorded by clamping the oocyte membrane potential at -120 mV and perfusing the oocytes with 5-HT or drug for 15-25 s and then washing with Ca\(^{2+}\)-Ringer’s solution for up to 45 s. Perfusion was controlled by gravity. Data was acquired digitally using Clampex 8.1 (Axon Instruments) and analyzed using Clampfit 8.1 (Axon Instruments) and SigmaPlot 5.0 (SPSS Science, Chicago, IL). Water-injected oocytes were assayed in parallel with SERT-injected oocytes to determine nonspecific effects on current by 5-HT, fluoxetine, and MMAI.

\textbf{Data Analysis}
$V_{\text{max}}$ and $K_{m}$ values in saturation experiments were calculated and exchange

$EC_{50}$ values were estimated using nonlinear curve-fitting analysis (Prism 3.0, GraphPad Software, San Diego, CA). All results were expressed as mean ± SEM for at least three experiments performed in duplicate or triplicate.
Results

**Kinetic analysis of 5-HT transport for wild-type and cross-species SERT chimeras**

Wild-type and chimeric SERTs demonstrated similar $K_m$ values for $[^3H] 5$-HT uptake (Table 1). In contrast, the 5-HT transport $V_{max}$ value for hSERT ($5.3 \pm 0.2 \times 10^{-17}$ mol/min/cell) was two-fold greater than dSERT ($2.3 \pm 0.1 \times 10^{-17}$ mol/min/cell) and the cross-species chimeras. The similar $K_m$ values suggest that both SERT species recognize 5-HT in a similar way. The differences in $V_{max}$ values between hSERT and dSERT could be the result of different cell surface expression levels in the transfected HEK-293 cells. To address this issue, we determined surface expression of SERTs by whole-cell binding assays carried out at 4 °C. $[^3H]$ citalopram and $[^3H]$ mazindol were used as radiolabeled ligands for hSERT and dSERT respectively. Fluoxetine (10 µM) was used to define nonspecific binding, and MPP$^+$ (400 µM) was used to bind SERT expressed only on the cell surface. Although we observed that 400 µM MPP$^+$ was transported by hSERT at 37 °C (Table 1), inward transport of this concentration of MPP$^+$ was not detected at 4 °C for hSERT (data not shown) or at any temperature for dSERT (see below, Table 1), thus, validating the use of MPP$^+$ to define surface expression. hSERT showed nearly eight-fold greater surface expression than dSERT, explaining in part the differences in $V_{max}$ between the two SERT species (Fig. 2A and B). $V_{max}$ values for hSERT and dSERT from these cells were used to approximate the 5-HT transport turnover number. Turnover number provides an opportunity for direct comparison between the two SERT species without concerns related to cell surface expression. Interestingly, the turnover number for dSERT was almost two-fold greater than hSERT.
(Fig. 2C) implying a potential distinction in the transport mechanism between hSERT and dSERT.

**MPP⁺ transport differences between human and *Drosophila* SERTs**

Although 5-HT appeared to be recognized similarly by hSERT and dSERT, we tested the hypothesis that other SERT substrates such as MPP⁺ and substituted-amphetamines would demonstrate species selectivity. Saturation studies in parental and chimeric SERTs were performed using the low-affinity substrate MPP⁺. This compound has been used extensively to study SERT function (Sitte et al., 2000; Scholze et al., 2000; Sitte et al., 2001) as well as other monoamine transporters (Sitte et al., 1998). MPP⁺ uptake at hSERT exhibited a $K_m$ value of 24 µM and $V_{max}$ value of $5.7 \times 10^{-17}$ mol/min/cell (Table 1). In contrast, neither dSERT nor the cross-species chimeras showed significant capacity to transport MPP⁺ under our experimental conditions. These findings suggest that despite similar recognition properties for 5-HT, hSERT and dSERT possess distinctions for molecular recognition and translocation of other substrates.

There are two possible explanations for the lack of inward MPP⁺ transport by dSERT and the cross-species chimeras: 1) dSERT and the cross-species chimeras lack the conformation or the binding site necessary to interact with MPP⁺, or 2) MPP⁺ binds but cannot be translocated through the transporter, acting like an antagonist instead of a substrate. To address this question, [³H] 5-HT uptake inhibition assays were performed in hSERT, dSERT, and the cross-species chimeras revealing that MPP⁺ can bind to all SERTs (Fig. 3). The lack of transport for MPP⁺ at dSERT and the chimeras suggests that MPP⁺ may have distinct interactions with the two SERT species homologues. For
example, MPP$^+$ binding to hSERT triggers transport, but that residues involved in promoting MPP$^+$ transport are absent or inaccessible in dSERT.

**Differences in amphetamine properties at the human and *Drosophila* SERTs**

To explore species-specific differences in amphetamine recognition and translocation at hSERT and dSERT, the capability of six amphetamine analogs to evoke 5-HT exchange was assessed (Fig. 1B and Table 2). The ability to induce exchange has been used as evidence that a compound is a substrate for transporters (Rudnick and Walls 1992 a, b). Exchange data revealed higher potency and efficacy of amphetamine-induced release through hSERT as compared to dSERT or the cross-species chimeras (Table 2, Fig. 4A). For example, the amphetamine analog MMAI was 6-fold more potent at hSERT (EC$_{50}$ = 530 ± 80 nM) as compared with dSERT (3050 ± 280 nM) and at least 24-fold more potent than at the cross-species chimeras (Table 2, Fig. 4A). Moreover, MMAI induced release of approximately 70% of the cytoplasmic 5-HT concentration in hSERT expressing cells, but this amphetamine analog only released approximately 30% and 10% of internal 5-HT in the chimeras and dSERT, respectively (Fig. 4A). Despite the lack of potency and efficacy for inducing 5-HT exchange at dSERT, MMAI and the other amphetamines (data not shown) were capable of inhibiting 5-HT transport at dSERT and the chimeras as assessed by [$^3$H] 5-HT uptake inhibition assays (Fig. 4B). The 5-HT transport inhibition studies further confirmed the fact that MMAI exhibited higher potency for hSERT than dSERT. The amphetamine derivative 3, 4-dichloroamphetamine (DCA) was the most potent compound tested at all SERTs. The EC$_{50}$ value for hSERT (70 ± 10 nM) was approximately 22-fold lower than dSERT (1340 ± 680 nM) or the cross-species
chimeras (Table 2). The analog 3, 4-difluoroamphetamine (DFA) that contains fluorines at the same positions as the chlorines in DCA, did not evoke 5-HT exchange through dSERT or the chimeras. The two para-substituted analogs 4-trifluoromethamphetamine (EC$_{50}$ = 270 ± 80 nM) and 4-methylthioamphetamine (EC$_{50}$ = 210 ± 80 nM) were nearly 12-fold more potent at hSERT than the meta-substituted derivative 3-methylthioamphetamine.

The data suggest that MMAI and the other amphetamines may not be effectively transported by dSERT and, thus, lack efficacy for inducing exchange. However, dSERT could have an impaired ability to outwardly transport substrates, so we tested the ability of the known substrate 5-HT to induce exchange at hSERT and dSERT. Similar to our findings with the amphetamine analogs, unlabeled 5-HT induced less release of internal substrate from dSERT compared to hSERT, suggesting that dSERT may have reduced capacity for reverse substrate transport (Fig. 4C).

To characterize further the differences in 5-HT release between hSERT and dSERT, we examined the rates and the turnover number for 5-HT release. First, we determined the initial rate of [3H] 5-HT release stimulated by unlabeled 5-HT (10 µM). hSERT demonstrated a faster rate of release (13.0 ± 3.0 x 10$^{-19}$ mol/min/cell) compared to dSERT (2.0 ± 0.6 x 10$^{-19}$ mol/min/cell)(data not shown). Release rates were normalized to the number of transporters at the cell surface to estimate the turnover number for exchange. Similar to inward 5-HT transport, the estimated turnover for exchange rate was statistically greater for dSERT. The estimated turnover rate for exchange was 0.66 ± 0.02 and 0.82 ± 0.01 molecules/min/transporter for hSERT and dSERT, respectively. Although 5-HT-induced release studies suggest a reduced
exchange capacity at dSERT, the estimated turnover number for exchange suggests that the apparent reduction in exchange capacity at dSERT is the result of lower cell surface expression.

Galli and coworkers demonstrated the regulation of human dopamine transporter (hDAT) surface expression by amphetamine (Saunders et al., 2000). Amphetamine promoted a loss of hDAT expression from the cell surface by redistribution of the transporter. 5-HT could induce a loss of dSERT surface expression during our preloading step. Loss of dSERT from the cell surface would lead to fewer transporters to participate in exchange and could explain in part the differences of substrate release between hSERT and dSERT. Preincubation with 5-HT at a concentration equivalent to that used for loading in our exchange experiments did not change cell surface expression of hSERT or dSERT as determined by cell surface binding experiments (data not shown). This result indicates that the differences between hSERT and dSERT for substrate release are not the result of changing cell surface expression.

Two-electrode voltage-clamp studies in oocytes injected with hSERT, dSERT, or H1-281D282-476 H477-638 cRNA were performed to determine if the amphetamines exhibited substrate-like properties for SERT (Fig. 5). Oocytes injected with either hSERT or dSERT cRNA exhibited a maximal inward current in the presence of 5-HT of 14.4 ± 1.7 nA and 84.0 ± 22 nA, respectively. Consistent with a SERT-associated effect, this current was blocked in the presence of fluoxetine. No 5-HT-induced currents were detected in oocytes injected with water (data not shown). MMAI induced an inward current in oocytes expressing hSERT comparable to that observed for 5-HT. In contrast, MMAI only induced a maximal current that was approximately 7% (6.0 ± 2.0 nA) of the
5-HT-induced current in oocytes expressing dSERT. Similar results showing reduced or undetectable amphetamine-induced currents at dSERT have been observed with other amphetamine analogs (data not shown). We also examined the H¹⁻²⁸¹D²⁸²₋⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimera for substrate-induced currents. Interestingly, this chimera, which demonstrated a dSERT-like phenotype for MPP⁺ uptake and amphetamine-induced exchange, had dSERT-like properties for 5-HT-induced currents, but exhibited MMAI-induced currents similar to hSERT (Fig. 5C). Although fluoxetine (20 μM) effectively inhibited substrate-induced currents at hSERT and dSERT, this concentration of inhibitor only partially inhibited the MMAI-induced currents at H¹⁻²⁸¹D²⁸²₋⁴⁷⁶H⁴⁷⁷⁻⁶³⁸. Whereas MMAI promoted substrate exchange and inhibited [³H]5-HT uptake with EC₅₀ and Kᵢ value of approximately 10 μM, the MMAI-induced current at the chimera reached maximal at 3 μM (48.1 ± 18.3 nA). This potency difference might be due to changes in SERT function resulting from varying expression levels in our two systems (Ramsey and DeFelice, 2002). Our data demonstrate that MMAI and most likely the other amphetamine analogs are not substrates for dSERT. Moreover, these results agree with our exchange data that implied a lack of efficient transport for amphetamines.
Discussion

Although several studies have explored the inward (Barker et al., 1999; Adkins et al., 2001; Sitte et al., 2001) and outward (Sitte et al., 2001; Sitte et al., 2000; Scholze et al., 2000; Rudnick and Walls 1992 a, b; Rudnick and Wall, 1993) transport process in SERT as well as other monoamine transporters (Pifl and Singer 1999), the molecular mechanisms of both processes remain poorly understood. Previous studies have exploited species-specific properties of SERTs to identify domains involved with transporter substrate and inhibitor recognition (Barker et al., 1994; Barker et al., 1998). For substrates, the SERT species-variants approach was used to explore the interaction of tryptamine analogs with the human and Drosophila SERTs (Adkins et al., 2001). Species-scanning mutagenesis implicated a single amino acid at position 95 for tryptamine recognition supporting the role of TMD I in the inward transport mechanism. Similarly, we used species-variants and cross-species chimeras to explore regions in the SERT involved in the inward and outward transport mechanism.

Although both hSERT and dSERT were able to translocate 5-HT, dSERT did not readily transport other SERT substrates such as MPP+ or amphetamines. $K_m$ values for 5-HT revealed similar relative affinities for both transporters. In contrast, the greater $V_{\text{max}}$ value for hSERT than dSERT may suggest differences in inward transport capacity or cell surface expression levels. Moreover, whole-cell binding studies confirmed higher hSERT expression on the cell surface than dSERT indicating that more human transporters are able to participate in uptake. We recognize that the whole-cell binding experiments require several assumptions, and that the method has potential caveats. For example, the ability of the ligands used (both labeled and unlabeled) to permeate
the membrane will vary depending upon the hydrophobicity of the specific compound. Our experiments were performed under assumed equilibrium (one hour incubation) and, thus, the hydrophobic ligands should sufficiently distribute to fully bind intracellular transporters. In addition, separation of bound and free radioligand can be problematic in whole-cell binding approaches. Our washes were completed within 10 sec which should minimize any loss of bound radioligand for the high-affinity radioligands used (K_d values = ~3 nM).

Studies were performed using MPP^+ to explore further species-selectivity for inward transport. Like previous reports, hSERT was able to transport MPP^+ (Sitte et al., 2000; Sitte et al., 2001). Interestingly, neither dSERT nor the cross-species chimeras transported MPP^+, suggesting species-distinction for substrate recognition and/or transport capacity between hSERT and dSERT. In further studies, MPP^+ inhibited 5-HT uptake in both parental and chimeric SERTs with similar potencies, demonstrating the ability of all SERT constructs to recognize MPP^+. These findings suggest species distinction for MPP^+ interactions between hSERT and dSERT. This molecule has substrate properties at hSERT (i.e. 5-HT), but interacts with dSERT like a transport blocker. Furthermore, our results from cross-species chimeras implicate TMDs V to IX of SERT in the inward transport mechanism but not necessarily in substrate recognition. Although our studies do not explore specific residues in this region, a study of rat DAT has suggested the importance of two serine residues localized in TMDs VII and XI for the inward transport of MPP^+ (Kitayama et al., 1993). Alanine substitutions for Ser^{350} and Ser^{353} at rDAT preferentially increased V_{max} for MPP^+ transport. Interestingly, Ser^{350} is conserved among the monoamine transporters. Whereas hSERT has shared identity
with rDAT at position 353, dSERT has an alanine residue at the corresponding position. Our results suggest the presence of specific residues in this region of SERT that are involved in the molecular mechanism of MPP⁺ uptake.

Another characteristic of SERT and other GAT/NET transport gene family members is the ability to outwardly transport substrate from the cytoplasm. A transporter-dependent release process has been demonstrated by changing transmembrane ion gradients (Pifl et al., 1997) or by the facilitated-exchange model (Wall et al., 1995; Johnson et al., 1998; Sitte et al., 1998). Our experiments focused on studying facilitated-exchange by SERT species-variants as a method to identify whether the substituted-amphetamines were transported. Our data revealed that the amphetamines were not effectively transported by dSERT and, hence, failed to induce substrate exchange. Amphetamine analogs blocked the uptake of 5-HT at dSERT, but were unable to induce substrate exchange or inward current. In addition, we observed that the substituted-amphetamines were less potent at dSERT as compared to hSERT suggesting species-specific differences in the recognition of the amphetamines exist that may or may not influence actual translocation. Results from the cross-species chimeras suggest that TMDs V to IX of SERT may be responsible for amphetamine recognition and transport. However, results from H¹-281D²⁸²-⁴⁷⁶H⁴⁷⁷-⁶³⁸ suggest that there are some distinctions for structural requirements needed for current activation as opposed to induced-exchange and MPP⁺ translocation. For example, the chimera did not appear to transport MPP⁺ and had minimal MMAI-induced exchange capacity, yet demonstrated MMAI-induced currents equivalent to those for 5-HT. This finding may
suggest that residues involved with gating SERT channel-like properties may reside in
the regions flanking TMDs V to IX.

Previous studies have demonstrated a channel mode of behavior for dSERT and
suggested that this mode may modulate substrate permeation at high extracellular
substrate concentration (Galli et al., 1997; Petersen and DeFelice, 1999). If such a
channel mode exists for dSERT, we would not expect exchange to occur because one
major distinction between transporters and channels is the inability of channels to carry
out substrate-induced exchange (Stein, 1986). Even in the case that dSERT may
behave as a channel, 5-HT exchange has been shown at high extracellular substrate
concentration (Petersen and DeFelice, 1999). dSERT may alternate between transport
and channel modes and is influenced by the environment (i.e. high sodium
concentration in the cytoplasm). The complex behavior of dSERT in this regard
warrants further study as it may have some contributory influence on potential
distinction in substrate recognition that modifies inward and outward transport
properties.

We also explored whether differences exist in the turnover numbers for the
inward and outward transport processes between hSERT and dSERT. Whole-cell
binding experiments demonstrated higher surface expression for hSERT than dSERT.
This finding explains the greater $V_{\text{max}}$ values for 5-HT uptake at hSERT than dSERT.
Calculation of the inward turnover number for dSERT revealed a two-fold greater value
than hSERT. Interestingly, the turnover numbers for exchange demonstrated a much
smaller difference between the species variants. These results suggest unequal
exchange between extracellular and intracellular substrates. hSERT and dSERT most
likely differ in the rate of inward transport relative to the reorientation of the transporter. The net inward transport rate for the two SERTs is influenced by many factors including the reorientation of the “empty” SERT to the outside. This reorientation rate could be altered by outwardly moving substrate during exchange, thus, possibly explaining the differences in inward and outward transport rates for hSERT and dSERT. A study performed in hDAT demonstrated that exchange between an external substrate and internal dopamine is unequal (Chen and Justice, 2000). Simultaneous monitoring of tyramine uptake and induced dopamine exchange revealed the initial exchange rate of internal dopamine is only 6% of the initial entry rate of external tyramine. These results confirm differences in the conformational requirements for inward and outward transport mechanisms that may be partially responsible for the asymmetric exchange between internal and external substrates.

In summary, our studies revealed major differences between hSERT and dSERT for substrate recognition and translocation. Our data demonstrated similar 5-HT kinetics for hSERT and dSERT, but major differences for other substrates might suggest that dSERT and hSERT possess fundamental differences for recognition of MPP⁺ and amphetamines that do not allow for translocation. Alternatively, hSERT and dSERT could possess differences affecting how all substrates are recognized and translocated. For 5-HT, these differences are not apparent based on our transport kinetic measures, but are revealed by other substrates. Our studies implicate the middle region of the SERT in substrate translocation through the membrane. Previously, a role of TMD I in the substrate permeation pathway has been defined (Barker et al., 1999). Recently, Ravna and Edvardsen constructed a hypothetical three-dimensional model of the
hSERT (Ravna and Edvardsen, 2001). Their arrangement of the TMDs places TMDs I and VII in the 5-HT permeation pathway. Our results lead to several questions about SERT structure and the molecular mechanism involved in substrate permeation. For example, identification of residues within TMDs V to IX involved in the species-specific properties may in part clarify the molecular mechanism of substrate transport. Moreover, this region may also interact with other TMDs to stabilize a specific conformation that is favorable for inward and outward transport. Future studies may provide insight about the inward and outward transport mechanisms at the same transporter species and will clarify molecular differences between both processes.
Acknowledgements

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Footnotes

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Figure 1. (A) Diagram of wild-type hSERT, dSERT, and cross-species chimeras. Two functional chimeras between hSERT (black) and dSERT (gray) were constructed as described in Materials and Methods. (B) Table of the amphetamine analogs used in these experiments. The table includes name, abbreviation, and the chemical structure.

Figure 2. Whole-cell radioligand binding in HEK 293 cells stably transfected with hSERT and dSERT. Assays were performed in six-well plates coated with poly-D-lysine as described in Materials and Methods. A saturating concentration of [³H] citalopram (20 nM) or [³H] mazindol (20 nM) was used for (A) hSERT and (B) dSERT, respectively. Nonspecific binding was defined as the binding of radiolabeled ligand in the presence of 10 µM fluoxetine. Internal binding was determined as the binding of [³H] ligand in the presence of 400 µM MPP⁺. Specific surface binding was calculated as (Total binding - binding in the presence fluoxetine) - (binding in the presence of MPP⁺ - binding in the presence of fluoxetine). (C) Turnover numbers for 5-HT transport at hSERT and dSERT were calculated as described in Materials and Methods. Bars represent the mean of three independent experiments ± SEM, *p <0.05 using a two-tailed Student’s t-test.

Figure 3. MPP⁺ inhibition of 5-HT uptake at wild type and chimeric SERTs. [³H] 5-HT uptake assays were performed as described in Material and Methods. Nonspecific uptake was determined with 10 µM fluoxetine. Evaluation of MPP⁺ potency at (■) hSERT (Kᵢ=250 ± 50µM), (▲) dSERT (Kᵢ=59 ± 6.0µM), (▼) H¹-118D¹19-627 (Kᵢ=73 ± 8.0µM), and (◆) H¹-281D²82-476H⁴77-638 (Kᵢ=56 ± 9.0µM). Data were plotted as percentage...
of specific 5-HT uptake. Results shown represent mean ± standard errors of triplicate determination and are representative of three independent experiments.

**Figure 4.** Effects of MMAI on the exchange of [³H] 5-HT release at hSERT and dSERT (A), uptake inhibition (B), or cold 5-HT-induced [³H] 5-HT exchange (C) in HEK 293 cells stably transfected with hSERT, dSERT, or cross-species chimeras. HEK 293 cells stably transfected with (■) hSERT, (▲) dSERT, (▼) H¹⁻¹¹₈D¹¹⁹⁻⁶²⁷, and (◆) H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ were loaded with [³H] 5-HT (50 nM) for five minutes (hSERT) or thirty minutes (dSERT and cross-species chimeras) and exchange induced by increasing concentration of unlabeled substrate as described in Materials and Methods. EC₅₀ values for MMAI and the other amphetamine derivatives are in Table 2. Stimulation of [³H] 5-HT exchange assay (C) by unlabeled 5-HT was performed at hSERT (EC₅₀= 7.7 ± 0.8 µM) and dSERT (6.1± 0.4 µM) as described in Materials and Methods. Results shown represent mean ± standard errors of triplicate determination and are representative of three independent experiments.

**Figure 5.** Two-electrode voltage-clamp studies in oocytes injected with SERT cRNAs. *Xenopus laevis* oocytes were injected with either hSERT (A), dSERT (B), or H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ (C) cRNA, and two-electrode voltage-clamp was performed as described in Materials and Methods. Oocytes were held at −120 mV and perfused with 5HT, MMAI, or fluoxetine as indicated. No substrate-induced currents were observed in water-injected oocytes (data not shown). Iₘₐₓ for hSERT was: 14.4 ± 1.7 nA (10 µM 5-HT, n=9), 14.1 ± 2.2 nA (3 µM MMAI, n=9), and 12.9 ± 2.1 nA (10 µM MMAI, n=7). Iₘₐₓ for dSERT was: 83.7 ± 21.5 nA (10 µM 5-HT, n=4), 5.5 ± 1.0 (3 µM MMAI, n=4), 6.0 ± 2.0
(10 μM MMAI, n=4), and 5.2 ± 2.0 (30 μM MMAI, n=4). $I_{\text{max}}$ for $\text{H}^{1-281}\text{D}^{282-476}\text{H}^{477-638}$ was: 50.5 ± 18.8 nA (10 μM 5-HT, n=4), 48.1 ± 18.3 (3 μM MMAI, n=4), 47.7 ± 17.6 (10 μM MMAI, n=4), and 42.4 ± 17.4 (30 μM MMAI, n=4). Data are representative of 4-9 oocytes from 2 or 3 different oocyte batches.
Table 1. $K_m$ and $V_{\text{max}}$ values for 5-HT and MPP$^+$ uptake in HEK 293 cells stably expressing parental and chimeric SERTs.

Kinetic values of 5-HT and MPP$^+$ uptake in HEK 293 cells stably transfected wild-type and chimera SERTs. Values represent mean ± standard errors for at least three independent experiments. ND: Not determined due to a lack of uptake at concentrations up to 1 mM.

<table>
<thead>
<tr>
<th></th>
<th>hSERT</th>
<th>dSERT</th>
<th>H$^{1-118}$D$^{119-627}$</th>
<th>H$^{1-281}$D$^{282-476}$H$^{477-638}$</th>
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<tbody>
<tr>
<td>5HT</td>
<td>$K_m$ (µM)</td>
<td>1.2 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>1.4 ± 0.2</td>
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<td>$V_{\text{max}}$ (mol/min/cell) x 10$^{-17}$</td>
<td>5.3 ± 0.2</td>
<td>2.3 ± 0.1</td>
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<tr>
<td>MPP$^+$</td>
<td>$K_m$ (µM)</td>
<td>24.2 ± 0.5</td>
<td>&gt;1000</td>
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<td></td>
<td>$V_{\text{max}}$ (mol/min/cell) x 10$^{-17}$</td>
<td>5.7 ± 0.6</td>
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Table 2. Estimated EC$_{50}$ values for [$^3$H] 5-HT exchange in stably transfected HEK-293 cells.

Amphetamine derivative EC$_{50}$ values (nM) for [$^3$H] 5-HT exchange in HEK 293 cells stably expressing hSERT, dSERT, or cross-species chimeras. EC$_{50}$ values represent mean ± S.E of three independent assays performed in triplicate. ND: Not determined due to lack of efficacy.

<table>
<thead>
<tr>
<th>Compound</th>
<th>hSERT (nM)</th>
<th>dSERT (nM)</th>
<th>H1-118D119-627 (nM)</th>
<th>H1-281D282-476H477-638 (nM)</th>
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<tr>
<td>5-methoxy-6-methyl-2-aminoindan</td>
<td>530 ± 80</td>
<td>3050 ± 280</td>
<td>35800 ± 2300</td>
<td>12600 ± 1060</td>
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<tr>
<td>3,4-dichloroamphetamine</td>
<td>70 ± 10</td>
<td>1340 ± 680</td>
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<td>3,4-difluoroamphetamine</td>
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<tr>
<td>4-trifluoromethylamphetamine</td>
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<td>6030 ± 2190</td>
<td>2150 ± 1100</td>
<td>6480 ± 1110</td>
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<tr>
<td>4-methylthioamphetamine</td>
<td>210 ± 80</td>
<td>19400 ± 5600</td>
<td>16600 ± 7300</td>
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<tr>
<td>3-methylthioamphetamine</td>
<td>2780 ± 680</td>
<td>16700 ± 6200</td>
<td>9600 ± 1570</td>
<td>11890 ± 1700</td>
</tr>
</tbody>
</table>
JPET #48751 Figure 1

A

hSERT

Introduced BsiWI site

NH₃

COOH

dSERT

Introduced BsiWI site

NH₃

COOH

B

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Abbreviation</th>
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<tr>
<td>3,4-methylenedioxyamphetamine</td>
<td>MDMA</td>
<td><img src="image" alt="MDMA" /></td>
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Figure 3

% of Specific 5-HT Uptake vs. Log [MPP⁺], M

- hSERT
- dSERT
- H¹-118 D¹¹⁹-6²⁷
- H¹-2⁸¹ D²⁸²-4⁷⁶ H⁴⁷⁷-6³⁸
A hSERT
oocyte held at −120 mV

5HT (10 μM) MMAl (3 μM) MMAl (10 μM) MMAl (30 μM) fluoxetine (20 μM)

20 nA
15 s

B dSERT
oocyte held at −120 mV

5HT (10 μM) MMAl (3 μM) MMAl (10 μM) MMAl (30 μM) MMAl (30 μM) fluoxetine (20 μM)

100 nA
15 s

C h1-281D282-476H477-638
oocyte held at −120 mV

5HT (10 μM) MMAl (3 μM) MMAl (10 μM) MMAl (30 μM) MMAl (30 μM) fluoxetine (20 μM)

100 nA
25 s