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-hydroxytryptamine (5-HT; serotonin) 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 8-fold lower than that of hSERT, dSERT does appear to have a 2-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, H1–118D119–627 and H1–281D282–476H477–638, were also unable to transport MPP$^+$, implicating the role of transmembrane domains 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 and Fornal, 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 a 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 a set of mechanisms, including biosynthetic enzymes, secretory proteins, ion channels, pre- and postsynaptic receptors, and transporters. The amphetamines [3,4-methylenedioxymethylamphetamine, 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 nonvesicular 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 (Corey et al., 1994; Demchushyn et al., 1994) revealed shared sequence identity with other members of the sodium- and chloride-dependent GABA/norepinephrine transporter gene family. Sequence analysis led to the prediction that SERTs, similar to other transporter homologs, possess 12 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
Distinct SERT Substrate Variants

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 Biosciences (Piscataway, NJ). [3H]Mazindol (21 Ci/mmol) and [3H]MPP+ (78 Ci/mmol) were obtained from PerkinElmer Life Sciences (Boston, MA). Fluoxetine, MPP+ and pargyline were purchased from Sigma-Aldrich (St. Louis, MO). Tranexamic acid, L-ascorbic acid, and penicillin, streptomycin, and L-glutamine were obtained from Sigma-Aldrich (St. Louis, MO). Mycillin, and G418 (600 mg/l) were purchased from GIBCO-BRL, Grand Island, NY. Cells were grown in a 37 °C humidified environment with 5% CO2.

HEK-293 cells stably expressing hSERT, dSERT, or chimeras were maintained in Dulbecco’s modified Eagle’s medium with 10% dialyzed fetal bovine serum supplemented with penicillin, streptomycin, l-glutamine, and G418 (600 mg/l). Cells were grown in a 37°C humidified environment with 5% CO2.

[3H]-Substrate Uptake Assays. For uptake inhibition assays, cells were plated as described for release assays. The time of assay, cells (1 × 10^6 cells per well) were washed once with Krebs/Ringer/Hepes (KRH) buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl2, 10 mM Hepes, 1.2 mM KH2PO4, 1.2 mM MgSO4, pH 7.4). Cells were incubated with increasing concentrations of substrate for 10 min at 37°C in KRH containing 15 mM glucose (15 ml final volume). Protein concentration was determined using the Bradford method (Bio-Rad, Hercules, CA), and suspensions were diluted in KRH buffer. The amount of remaining radiolabeled substrate was then determined using a PerkinElmer TopCount-NXT Microplate Scintillation and Luminescence Counter. Saturation assays on the substituted amphetamines that 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. The time of assay, cells (1 × 10^6 cells per well) were washed once with Krebs/Ringer/Hepes (KRH) buffer, incubated with increasing concentration of drug at 37°C for 10 min, and then treated with [3H]5-HT (20 nM). Cells were incubated another 10 min 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 × 20 mm) and incubated at 37°C for 2 days (approximately 90% confluent). Cells were resuspended in Krebs/glucose (15 ml final volume). Protein concentrations were determined using the Bradford method (Bio-Rad, Hercules, CA), and suspensions were diluted to 0.5 mg/ml with Krebs/glucose buffer. 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 5 min (hSERT) or 30 min (dSERT, H118D119 → D120, H118D119 → D281, and H118D119 → D638) 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 10 min, 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 (1 × 10^6 cells per well)
precoated with poly-D-lysine. The day after plating, cells were washed once with KRH. A saturating concentration (20 nM) of \[^{3}H\]citalopram or \[^{3}H\]mazindol was used as a radiolabeled ligand for hSERT and dSERT, respectively. Radiolabeled ligand and cells were incubated at 4 °C for 1 h. After incubation, cells were rapidly washed twice with 1000 μl of KRH, solubilized with 10% sodium dodecyl sulfate solution, and transferred into a scintillation vial. \[^{3}H\]-Ligand was determined using a Beckman LS 1801 liquid scintillation counter (Beckman Coulter, Fullerton, 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 and Science University). \({ }^{1-281}D^{282-476}H^{477-638}OTV\) was constructed by subcloning \(D^{282-476}\) into hSERT/OTV using the restriction sites described in this paper. Construction was confirmed enzymatically with \(Bgl\)II and \(Afl\)III. All cDNAs were linearized with \(Xba\)I. hSERT, dSERT, and \({ }^{1-281}D^{282-476}H^{477-638}\) cRNA was transcribed using the T7 mMessage mMachine In Vitro
Transcription Kit (Ambion, Inc., Austin, TX). Defolliculated stage V and VI oocytes were injected with 45 to 60 ng of either hSERT, dSERT, or H1-281D282–476 H11006–638 cRNA and maintained in Ca2+-Ringer’s solution (2 mM KCl, 5 mM MgCl2, 96 mM NaCl, 5 mM HEPES, and 0.6 mM CaCl2, pH 7.6) supplemented with 5% horse serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Invitrogen) at 18°C. Recordings were performed 6 to 7 days 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, Union City, CA). Glass microelectrodes were pulled to a resistance of 0.2 to 1.5 MΩ and filled with 3 M KCl. Recording solutions consisted of room-temperature Ca2+-Ringer’s solution with 5-HT, fluoxetine, or 5-methoxy-6-methyl-2-aminoindole (MAA) 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 to 25 s, and then washing with Ca2+-Ringer’s solution for up to 45 s. Perfusion was controlled by gravity. Data were 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 MAA.

Data Analysis. Vmax and Km values in saturation experiments were calculated, and exchange EC50 values were estimated using nonlinear curve-fitting analysis (Prism 3.0; GraphPad Software Inc., San Diego, CA). All results were expressed as mean ± S.E.M. 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 Kmax values for [3H]5-HT uptake (Table 1). In contrast, the 5-HT transport Vmax value for hSERT (5.3 ± 0.2 × 10−17 mol/min/cell) was 2-fold greater than that for dSERT (2.3 ± 0.1 × 10−17 mol/min/cell) and the cross-species chimeras. The similar Kmax values suggest that both SERT species recognize 5-HT in a similar way. The differences in Vmax 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 and Table 1), thus validating the use of MPP+ to define surface expression. hSERT showed nearly 8-fold greater surface expression than dSERT, explaining in part the differences in Vmax between the two SERT species (Fig. 2, A and B). Vmax 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 2-fold greater than that for 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 (Scholze et al., 2000; Sitte et al., 2000, 2001) as well as other monoamine transporters (Sitte et al., 1998). MPP+ uptake at hSERT exhibited a Km value of 24 μM and a Vmax value of 5.7 × 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 homologs. For example, MPP+ binding to hSERT triggers transport, but 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; Table 2). The ability to induce exchange has been used as evidence that a compound is a substrate for transporters (Rudnick and Wall, 1992a,b). Exchange data revealed higher potency and

| Table 1 |
|------------------|------------------|------------------|------------------|
| Km (μM) | Vmax (mol/min/cell) × 10−17 | Km (μM) | Vmax (mol/min/cell) × 10−17 |
| hSERT | dSERT | hSERT | dSERT |
| 5-HT | 1.2 ± 0.2 | 0.9 ± 0.2 | 1.4 ± 0.2 | 0.8 ± 0.1 |
| MPP+ | 5.3 ± 0.2 | 2.3 ± 0.1 | 3.3 ± 0.1 | 2.4 ± 0.1 |
| N.D. | N.D. | N.D. | N.D. |

N.D., not determined due to a lack of uptake at concentrations up to 1 mM.
efficacy of amphetamine-induced release through hSERT as compared with dSERT or the cross-species chimeras (Table 2; Fig. 4A). For example, the amphetamine analog MMAI was 6-fold more potent at hSERT (EC\textsubscript{50} = 530 ± 80 nM) 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 for dSERT. The amphetamine derivative 3,4-dichloroamphetamine was the most potent compound tested at all SERTs. The EC\textsubscript{50} value for hSERT (70 ± 10 nM) was approximately 22-fold lower than that for dSERT (1340 ± 680 nM) or the cross-species chimeras (Table 2). The analog 3,4-difluoroamphetamine, which contains fluorines at the same positions as the chlorines in 3,4-dichloroamphetamine, did not evoke 5-HT exchange through dSERT or the chimeras. The two para-substituted analogs 4-trifluoromethylamphetamine (EC\textsubscript{50} = 270 ± 80 nM) and 4-methylthioamphetamine (EC\textsubscript{50} = 210 ± 80 nM) were nearly 12-fold more potent at hSERT than was 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; therefore, 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 with 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
S.E. of three independent assays performed in triplicate.

induced currents at dSERT have been observed with other results showing reduced or undetectable amphetamine-

maximal current that was approximately 7% (6.0 inward current in oocytes expressing hSERT comparable to cyctes injected with water (data not shown). MMAI induced an associated effect, this current was blocked in the presence of and 84.0 maximal inward current in the presence of 5-HT of 14.4 injected with either hSERT or dSERT cRNA exhibited a max-

imated substrate-like properties for SERT (Fig. 5). Oocytes in-
dermed to determine whether the amphetamines exhib-
hover rate for exchange was 0.66 rate was statistically greater for dSERT. The estimated turn-

over 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 × 10⁻¹⁵ mol/min/cell) compared with dSERT (2.0 ± 0.6 × 10⁻¹⁵ 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 turn-

over rate for exchange was 0.66 ± 0.02 and 0.82 ± 0.01 molecules/min/transporter for hSERT and dSERT, respect-

ively. Although 5-HT-induced release studies suggest a re-
duced 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 (Saunders et al., 2000) demonstrated the regulation of human dopamine transporter (hDAT) surface expression by amphetamine. 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 chang-
ing cell surface expression.

Two-electrode voltage-clamp studies in oocytes injected with hSERT, dSERT, or H¹–281D²82–476H¹⁷⁷–638 cRNA were performed to determine whether 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. Sim-

ilar results showing reduced or undetectable amphetamine-

induced currents at dSERT have been observed with other amphetamine analogs (data not shown). We also examined the H¹–281D²82–476H¹⁷⁷–638 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-
duced currents but exhibited MMAI-induced currents similar to those of 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 H¹–281D²82–476 H¹⁷⁷–638. Whereas MMAI promoted substrate exchange and inhibited [3H]5-HT uptake with EC₅₀ and Kᵣ value of approxi-

mately 10 μM, the MMAI-induced current at the chimera reached maximum 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 (Ram-
sey 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, which 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 (Scholze et al., 2000; Sitte et al., 2000, 2001; Rudnick and Wall, 1992a,b, 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 transport-er substrate and inhibitor recognition (Barker et al., 1994, 1998). For substrates, the SERT species variants approach was used to explore the interaction of tryptamine analogs with the human and Drosophila SERT's (Adkins et al., 2001). Species-scanning mutagenesis implicated a single amino acid at position 95 for tryptamine recognition, sup-
porting the role of TMD I in the inward transport mecha-
nism. 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 sub-

strates such as MPP⁺ or amphetamines. Kᵥ values for 5-HT revealed similar relative affinities for both transporters. In contrast, the greater Vᵥₘₐₓ value for hSERT than for 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 expression, 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 (1-h 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 s, 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. As in previous reports, hSERT was able to transport MPP^+ (Sitte et al., 2000, 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 additional 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 Ser350 and Ser353 at rat DAT preferentially increased V_max for MPP^+ transport. Interestingly, Ser350 is conserved among the monoamine transporters. Whereas hSERT has shared identity with rat DAT 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 GABA/norepinephrine transporter 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 with hSERT, suggesting that 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 H1–284D282–476 H177–638 suggest that there are some distinctions in structural requirements needed for current activation as opposed to induced exchange and MPP⁺ translocation. For example, the chimera did not appear to transport MPP⁺ demonstrated higher surface expression for hSERT than for dSERT. Whole-cell binding experiments between hSERT and dSERT. Whole-cell binding experiments demonstrated higher surface expression for hSERT than for dSERT. This finding explains the greater \( V_{\text{max}} \) values for 5-HT uptake at hSERT than at dSERT. Calculation of the inward turnover number for dSERT revealed a 2-fold greater value than for 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 that 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 (2001) constructed a hypothetical three-dimensional model of the hSERT. 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.

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References

Adkins EM, Barker EL, and Blakely RD (2001) Interactions of tryptamine deriva-
tives with serotonin transporter species variants implicate transmembrane do-
8378.
Barker EL and Blakely RD (1995) Norepinephrine and serotonin transporters: molec-
ular targets for antidepressant drugs, in Psychopharmacology: The Fourth
Barker EL, Perlman MA, Adkins EM, Houlihan WJ, Pristupa ZB, Niznik HB, and
Blakely RD, Berson HE, Fremeau RT Jr, Caron MG, Peek MM, Price HK, and
Carvelli L, Javitch JA, and Galli A (2000) A putative three-dimensional arrangement of
the human serotonin transporter transmembrane helices: a tool to aid experimen-
tal studies. J Mol Graph Model 20:133–144.
Demchyshyn LL, Pristupa ZB, Sugamori KS, Barker EL, Blakely RD, Wolfgang WJ,
and Forte MA, and Niznik HB (1994) Cloning, expression and localization of a chloride-
facilitated, cocaine-sensitive serotonin transporter from Drosophila melanogaster.
Ferrer JV and Javitch JA (1998) Cocaine alters the accessibility of endogenous
cysteines in putative extracellular and intracellular loops of the human dopamine
Galli A, Petersen CF, deBlauquiere M, Blakely RD, and DeFelice LJ (1997) Drosophila
serotonin transporters have voltage-dependent uptake coupled to a serotonin-
Gu H, Wall SC, and Rudnick G (1994) Stable expression of biogenic amine trans-
porters reveals differences in inhibitor sensitivity, kinetics and ion dependence. J
Jaco de BL and Fornal CA (1988) Serotonin and behavior: a general hypothesis, in
Progress in Psychopharmacology: The Fourth Generation of Progress (Bloom F and Kupfer D
Johnson RA, Eshleman AJ, Meyers T, Neve KA, and Janowsky A (1998) ‘‘P’’-sub-
stituted and cell-specific effects of uptake inhibitors on human dopamine and
Moore KR and Blakely RD (1994) Restriction site-independent formation of chimeras
from homologous neurotransmitter transporter cDNAs. Biotechniques 17:130–
135.
Petersen CF and DeFelice LJ (1999) Ion interactions in the Drosophila serotonin
transporter identify it as a serotonin channel. Nat Neurosci 2:605–610.
or norepinephrine transporter transfected cells questions the hypothesis of facil-
kinase C activation regulates human serotonin transporters in HEK-293 cells via
Ramamoorthy S, Bauman AL, Moore KR, Han H, Yang Feng T, Chang AS, Gainath-
py V, and Blakely RD (1993) Antidepressant- and cocaine-sensitive human sero-
tonin transporter: molecular cloning, expression and chromosomal localization.
Proc Natl Acad Sci USA 90:2542–2546.
Raney IS and DeFelice LJ (2002) Serotonin transporter function and pharmacology
are sensitive to expression level. J Biol Chem 277:14475–14482.
serotonin transporter transmembrane helices: a tool to aid experimen-
tal studies. J Mol Graph Model 20:133–144.
kinetic determinations of stoichiometry, in Methods in Enzymology: Neurotrans-
Rudnick G and Wall SC (1993) p-Chloroamphetamine induces serotonin release
Rudnick G and Wall SC (1992b) The molecular mechanism of ‘‘estacy’’ (3,4-
methylenedioxyamphetamine (MDMA): serotonin transporters are targets
Rudnick G and Wall SC (1993) Non-neurotoxic amphetamine derivatives release
Saunders C, Ferrer JV, Shi L, Chen J, Merrill G, Lamb ME, Leeb-Lundberg LM,
dopamine transporter activity: an internalization-dependent and cocaine-sensitive
meditated release: a superfusion study on human embryonic kidney cells stably
analysis of inward and outward transporter rates in cells stably expressing the
cloned human serotonin transporter: inconsistencies with the hypothesis of facil-
meditated release, transport rates, and charge transfer induced by amphetamine,
tyramine and dopamine in mammalian cells transfected with the human dopa-
rier-mediated efflux in human embryonic kidney 293 cells stably expressing the
Stein WD (1986) Transport and Diffusion Across Cell Membranes, pp 231–237,
Wall SC, Gu H, and Rudnick G (1995) Biogenic amine flux mediated by cloned
transporters stably expressed in cultured cell lines: amphetamine specificity for
393:118–124.

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