Dopamine Transporter Amino and Carboxyl Termini Synergistically Contribute to Substrate and Inhibitor Affinities*

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Extracellular dopamine and serotonin concentrations are determined by the presynaptic dopamine (DAT) and serotonin (SERT) transporters, respectively. Numerous studies have investigated the DAT and SERT structural elements contributing to inhibitor and substrate binding. To date, crystallographic studies have focused on conserved transmembrane domains, where multiple substrate binding and translocation features are conserved. However, it is unknown what, if any, role the highly divergent intracellular N and C termini contribute to these processes. Here, we used chimeric proteins to test whether DAT and SERT N and C termini contribute to transporter substrate and inhibitor affinities. Replacing the DAT N terminus with that of SERT had no effect on DA transport $V_{\text{max}}$ but significantly decreased DAT substrate affinities for DA and amphetamine. Similar losses in uptake inhibition were observed for small DAT inhibitors, whereas substituting the DAT C terminus with that of SERT affected neither substrate nor inhibitor affinities. In contrast, the N-terminal substitution was completely tolerated by the larger DAT inhibitors, which exhibited no loss in apparent affinity. Remarkably, all affinity losses were rescued in DAT chimeras encoding both SERT N and C termini. The sensitivity to amino-terminal substitution was specific for DAT, because replacing the SERT N and/or C termini affected neither substrate nor inhibitor affinities. Taken together, these findings provide compelling experimental evidence that DAT N and C termini synergistically contribute to substrate and inhibitor affinities.

Psychostimulant drugs include therapeutics for multiple psychiatric disorders, such as depression and attention deficit hyperactivity disorder, as well as drugs of abuse (1–4). Determining how these drugs interact with their molecular targets is essential for understanding psychostimulant addiction, as well as psychiatric treatment. The monoamine transporters DAT and SERT are the primary targets for a variety of psychostimulants, such as cocaine and amphetamines (AMPH, methamphetamine, and 3,4-methylenedioxymethamphetamine), as well as the SSRI and norepinephrine-dopamine reuptake inhibitor classes of antidepressants (1–3, 5). Cocaine and SSRI/nor-epinephrine-dopamine reuptake inhibitors inhibit these transporters by directly blocking DA and 5HT reuptake, whereas amphetamine and its congeners are competitive DAT and SERT substrates. Once in the cytosol, amphetamines mediate monoamine release from vesicles in a vesicular monoamine transporter-dependent manner (6) and drive their efflux via their respective plasma membrane transporters (7).

DAT and SERT belong to the SLC6 family of Na$^+$- and Cl$^-$-dependent solute carriers, which are topologically similar, having 12 transmembrane domains and intracellular N and C termini (1–4). Crystal structures of the SLC6 bacterial homologs leucine transporter (8), dDAT (9), and more recently human SERT (10) have confirmed the predicted topologies of SLC6 transporters, shed light on the mechanism of substrate translocation, and better mapped inhibitor/substrate binding sites. Although critical in understanding transporter structure and function, these transporter crystallographic studies lack structural insights into the N terminus and much of the C terminus, because of their highly disordered properties. Mutagenesis studies have revealed that DAT and SERT intracellular N- and C-terminal domains contribute to transporter regulated internalization (11–17), post-endocytic trafficking (16, 18), biosynthetic trafficking (16, 19–22), and AMPH-stimulated substrate efflux (23–27). Moreover, DAT and SERT N- and C-terminal coding variants have been identified in attention deficit hyperactivity disorder and autism spectrum disorder patients (28–32), further implicating these regions as important for transporter function and necessitating the need for greater understanding of how they may contribute to transporter mechanisms of action. Computational modeling of the DAT N

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‡ The abbreviations used are: DAT, dopamine transporter; SERT, serotonin transporter; DA, dopamine; 5HT, serotonin; AMPH, amphetamine; SSRI, selective serotonin reuptake inhibitor; SLC6, solute carrier 6; dDAT, Dro-sophila dopamine transporter; PIP$_2$, phosphatidylinositol 4,5-bisphosphate; ANOVA, analysis of variance; MPH, methylphenidate; KRH, Krebs-Ringers-HEPES; β-CIT, (−)-2β-carbomethoxy-3β-(4-iodophenyl)tropane; β-CFT, (−)-2β-carbomethoxy-3β-(4-fluorophenyl)tropane.
terminus (33, 34), and both SERT N and C termini (35), has shed light onto their secondary structure. Moreover, these studies have raised the possibility that these domains may interact with the plasma membrane and, potentially, each other. However, there remains a dearth of experimental data testing whether or not these domains contribute to substrate and inhibitor affinities. In the current study, we asked whether DAT and SERT N and C termini influenced substrate and inhibitor affinities. To address this question, we generated DAT/SERT chimeras in which the N terminus, C terminus, or both termini are exchanged between their respective DAT or SERT core proteins. DAT and SERT transmembrane cores share 52% sequence identity; however, their N and C termini are highly divergent (17 and 27% sequence identity, respectively), thus providing an effective strategy to test whether these intracellular domains differentially contribute to substrate or inhibitor affinities.

Results

SLC6 crystallographic studies have shed considerable light on both substrate translocation mechanisms and inhibitor binding sites (8–10, 38). However, it remains uncertain whether transporter intracellular N and C termini contribute to these fundamental transporter properties. To test the hypothesis that these cytoplasmic domains influence the transport mechanism and inhibitor binding, we created DAT/SERT chimeras in which the N terminus, C terminus, or both termini are exchanged between their respective DAT or SERT core proteins. DAT and SERT transmembrane cores share 52% sequence identity; however, their N and C termini are highly divergent (17 and 27% sequence identity, respectively), thus providing an effective strategy to test whether these intracellular domains differentially contribute to substrate or inhibitor affinities.

Figure 1A, DAT/SERT chimera schematic. DAT “core” and SERT “core” chimeric proteins were designed as depicted. DAT regions are indicated in red, and SERT regions are indicated in cyan. B, DAT and SERT N and C termini are highly divergent. Sequence alignments of DAT and SERT N and C termini. Conserved residues (by identity, charge, or size) are indicated in red.

DAT/N-SERT/DAT
DAT(1-78)/N-SERT(60-620)

N-SERT/DAT
N-SERT(1-78)/DAT(60-620)

DAT/C-SERT
DAT(1-583)/C-SERT(601-630)

SERT/C-DAT
SERT(1-500)/C-DAT(584-620)

SERT/DAT/SERT
SERT(1-78)/DAT(60-620)/SERT(601-630)

DAT/SERT/DAT
DAT(1-59)/SERT(79-630)/DAT(584-620)

DAT/C-SERT, and both SERT N and C termini (35), has shed light onto their secondary structure. Moreover, these studies have raised the possibility that these domains may interact with the plasma membrane and, potentially, each other. However, there remains a dearth of experimental data testing whether or not these domains contribute to substrate and inhibitor affinities. In the current study, we asked whether DAT and SERT N and C termini influenced substrate and inhibitor affinities. To address this question, we generated DAT/SERT chimeras in which the N terminus, C terminus, or both termini are exchanged between their respective DAT or SERT core proteins. DAT and SERT transmembrane cores share 52% sequence identity; however, their N and C termini are highly divergent (17 and 27% sequence identity, respectively), thus providing an effective strategy to test whether these intracellular domains differentially contribute to substrate or inhibitor affinities.

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DA Transporter Termini Impact Substrate Affinity

FIGURE 2. DAT core chimera uptake kinetics and surface expression. A, [3H]DA uptake assays. Saturation uptake kinetics were measured as described under “Experimental Procedures,” in SK-N-MC cells stably expressing either WT DAT (black), N-SERT/DAT (cyan), DAT/C-SERT (blue), or SERT/DAT/SERT (red). The average data are presented ± S.E. (n = 11–16). B–D, surface biotinylation assays. Surface levels for the indicated constructs were determined by surface biotinylation, as described under “Experimental Procedures.” Surface protein levels for the indicated constructs were determined relative to the total amount of DAT chimeras expressed (p = 0.72, Student’s t test, n = 3–4). C, WT DAT versus DAT/C-SERT total protein levels, calculated as the sum of biotinylated (surface) and supernatant (intracellular) fractions, * significantly different from WT DAT (p < 0.04, Student’s two-tailed t test, n = 3–4). Inset, representative immunoblot for B and C. D, N-SERT/DAT and SERT/DAT/SERT surface protein expression, normalized to actin. ***, significantly different from N-SERT/DAT (p < 0.001), Student’s two-tailed t test, n = 3. Inset, representative immunoblot. (Note: boxed bands were digitally extracted from a single blot exposure, probed for both the chimeras and actin, in parallel.)

the DAT N terminus with the SERT N terminus significantly decreased the apparent substrate affinity, as reflected by an increased $K_m$ for DA (WT DAT: $1.1 ± 0.1 \mu M$; N-SERT/DAT: $2.2 ± 0.4 \mu M$, n = 11–16; Table 1), whereas substituting the DAT C terminus with that of SERT (DAT/C-SERT) had no effect on apparent DA affinity compared with WT DAT (p = 0.99; Table 1). Surprisingly, replacing both DAT N and C termini with those of SERT restored the $K_m$ for WT DAT levels, thereby rescuing the loss of apparent affinity induced by substituting the DAT N terminus alone (Table 1). We next asked whether the N terminus contributes to apparent substrate affinity in general, or to apparent DA affinity specifically, by measuring the sensitivity of each chimera to AMPH, a competitive DAT substrate. As illustrated in Table 1, similar to our findings with DA, N-SERT/DAT exhibited a significant increased $K_i$ for AMPH, compared with WT DAT, indicating a loss of apparent substrate affinity, whereas DAT/C-SERT showed no change in AMPH sensitivity compared with WT. Moreover, SERT/DAT/SERT exhibited a significant decrease in $K_i$ for AMPH (52.1 ± 11.4 nM, n = 11, p < 0.05), indicating an increased apparent affinity.

Given that DAT N and C termini differentially impacted apparent substrate affinity, we next extended our analyses to test whether these domains also contribute to competitive inhibitor affinities. We first focused on cocaine and other tropine-derived congeners. Despite the impact that N-terminal substitution imposed upon apparent substrate affinity, none of the DAT core chimeras exhibited a significant difference in $K_i$ values for cocaine, as compared with WT DAT (Table 1). In contrast, similar to what we observed for DAT substrates, N-SERT/DAT exhibited significantly lower apparent affinity than WT DAT for the high affinity DAT inhibitor β-CFT (WIN35,428; n = 4). Moreover, substituting the SERT C terminus had no effect on apparent β-CFT affinity, and substituting both the DAT N and C terminus rescued the loss in apparent affinity observed with the N-terminal substitution (Table 1).

We further tested whether the apparent affinity of another high affinity tropane, β-CIT (RTI-55), was similarly impacted by the DAT N-terminal substitution. Interestingly, despite the structural similarity between β-CIT and β-CFT, N-SERT/DAT exhibited no difference in apparent affinity for β-CIT as compared with WT DAT (Table 1, Student’s two-tailed t test, p = 0.33, n = 3–7). Cocaine is a relatively low affinity inhibitor with equimolar potency across monoamine transporters, whereas both β-CFT and β-CIT are non-selective, high affinity inhibitors for both DAT and SERT. Therefore, we next tested whether methylphenidate (MPH) and GBR12909, high affinity inhibitors with selectively for DAT over SERT, were similarly affected by the DAT N- and C-terminal substitutions. Similar to what we observed with DAT substrates, N-SERT/DAT exhibited significantly decreased sensitivity to MPH, whereas MPH apparent affinities for DAT/C-SERT were not significantly different from WT DAT (p = 0.99) and SERT/DAT/SERT rescued the loss in sensitivity observed for N-SERT/DAT (Table 1). In contrast, substituting DAT N and C termini with SERT termini had no effect on DAT core chimera sensitivity to the high affinity DAT inhibitor, GBR12909 (p = 0.37).

We next tested whether reciprocal DAT substitutions onto the SERT core would similarly impact apparent substrate and inhibitor affinities. WT SERT and SERT core chimeras exhibited robust, saturable 5HT uptake when expressed in SK-N-MC cells (Fig. 3A), and no significant differences in $V_{max}$ values were observed among the chimeras as compared with WT SERT, although N-DAT/SERT exhibited a trend toward decreased 5HT transport $V_{max}$ (p = 0.07). Assessment of 5HT $K_m$ values revealed no differences between WT SERT and any of the SERT core chimeras (Table 2), suggesting that, unlike DAT, the SERT N terminus does not
TABLE 1
DA Transporter Termini Impact Substrate Affinity
The values are in nM unless otherwise indicated. n = 11–17 for [3H]DA kinetics and 3–11 for K_i values. ND, not determined.

| [3H]DA uptake kinetics | WT DAT | N-SERT/DAT | DAT/C-SERT | SERT/DAT/SERT |
|------------------------|--------|------------|------------|--------------|
| V_max (pmol/min/mg protein) | 45.0 ± 8.8 | 55.0 ± 10.9 | 11.7 ± 2.7^a | 7.8 ± 1.5^a |
| K_m (μM) | 1.1 ± 1.0 | 2.2 ± 0.4^a | 1.1 ± 0.2 | 1.2 ± 0.2^a |

K_i values:
- Amphetamine: 348.9 ± 69.2 vs. 672.6 ± 141.4 μM
- Cocaine: 289.9 ± 63.9 vs. 214.0 ± 50.8 μM
- β-CFT: 18.2 ± 4.1 vs. 657.7 ± 15.7 μM
- β-CIT: 0.42 ± 0.14 vs. 0.20 ± 0.02 μM
- Methylphenidate: 63.5 ± 12.0 vs. 157.9 ± 31.7 μM
- GBR12909: 2.1 ± 0.22 vs. 2.8 ± 0.6 μM

^a Significantly different from WT DAT, p < 0.01, one-way ANOVA with Dunnett's multiple comparison test.
^b Significantly different from WT DAT, p < 0.001, one-way ANOVA with Bonferroni's multiple comparison test.
^c Significantly different from WT DAT, p < 0.01, one-way ANOVA with Bonferroni's multiple comparison test.
^d Significantly different from WT DAT, p < 0.05, one-way ANOVA with Bonferroni’s multiple comparison test.

TABLE 2
SERT core chimeras [3H]5HT uptake kinetics and inhibitor K_i values
The values are in nM unless otherwise indicated. n = 5–6 for [3H]5HT kinetics and 4–7 for K_i values.

| [3H]5HT uptake kinetics | WT SERT | N-DAT/SERT | SERT/C-DAT | DAT/SERT/DAT |
|------------------------|---------|------------|------------|--------------|
| V_max (pmol/min/mg protein) | 50.2 ± 8.6 | 17.7 ± 1.5 | 49.7 ± 9.7 | 39.3 ± 10.1 |
| K_m (μM) | 0.75 ± 0.1 | 0.71 ± 0.1 | 0.62 ± 0.1 | 0.89 ± 0.1 |

K_i values:
- Fenfluramine (μM): 1.5 ± 0.4 vs. 1.8 ± 0.6 μM
- Cocaine: 153.3 ± 16.2 vs. 160.0 ± 41.6 μM
- β-CIT: 0.28 ± 0.0 vs. 0.35 ± 0.05 μM
- Escitalopram: 1.4 ± 0.4 vs. 1.3 ± 0.9 μM
- Imipramine: 6.4 ± 0.7 vs. 8.7 ± 2.3 μM

^a Significantly different from WT SERT, p < 0.05, one-way ANOVA with Bonferroni's multiple comparison test.
^b Significantly different from WT SERT, p < 0.01, one-way ANOVA with Bonferroni’s multiple comparison test.
^c Significantly different from N-SERT/DAT, p < 0.05, one-way ANOVA with Bonferroni’s multiple comparison test.
^d Significantly different from N-SERT/DAT, p < 0.05, one-way ANOVA with Bonferroni’s multiple comparison test.

contribute to apparent substrate affinity. To test whether this is specific to SHT or applies to other SERT substrates, we measured the apparent affinity for fenfluramine, a SERT-selective amphetamine derivative. As seen in Table 2, substituting the DAT N termini, C termini, or both had no effect on the SERT core chimera fenfluramine K_i values, as compared with WT SERT. We similarly tested a panel of chemically diverse SERT inhibitors, including the tropane compounds cocaine and β-CIT, the SSRI escitalopram, and the tricyclic antidepressant, imipramine. Replacing the DAT N and/or C termini onto SERT had no effect on the potency of these compounds, as compared with WT SERT (Table 2), suggesting that although the DAT N terminus contributes to apparent substrate affinity and potency for a subclass of inhibitors, SERT can tolerate N-terminal substitutions without compromising either apparent substrate or inhibitor affinities.

Discussion

Monoamine transporter N and C termini encode multiple motifs critical for biosynthesis (19–22, 39), surface regulation (11–13), and AMPH-stimulated substrate efflux (23–27). Given that coding variants in both the DAT and SERT terminal domains have been independently reported in patients with neuropsychiatric disorders (28–30, 32), understanding how these domains may also influence substrate and inhibitor binding is critical. DAT N-terminal residues interact with intracellular domains that contribute to an intracellular transport gating mechanism, which modulates substrate translocation (8, 25, 26, 34, 40). In particular, Arg^60 (41), Thr^62 (26), and Arg^61 (34) interact with intracellular loops to help stabilize the DAT outward (Arg^60 and Thr^62) and inward (Arg^61) facing conformations. Arg^60 and Thr^62 are maintained in our chimeras, as they are conserved between DAT and SERT. However, Arg^61 is not conserved between DAT and SERT, and observed differences in substrate/inhibitor apparent affinities may be due to a loss of Arg^61 influence on gating. This putative change in gating may be translated to the substrate binding pocket and thereby result in decreased apparent affinities for certain DAT substrates and inhibitors. An R51W coding variant identified in autism spectrum patients was recently reported (32), which results in reduced DA efflux capacity, consistent with a role for Arg^51 in transitioning between inward and outward facing conformations. Such results further raise the possibility that altered N-terminal mobility may have broader functional implications in neuropsychiatric disorders.

Our results using DAT/SERT chimeric proteins revealed that the DAT N terminus influences the apparent affinity of substrates and a subset of DAT inhibitors, whereas SERT function and inhibition was insensitive to N- and C-terminal substitutions. N-SERT/DAT exhibited reduced apparent affinity for DAT substrates DA and AMPH, as well as the inhibitors MPH and β-CFT. Interestingly, the N terminus did not contribute to apparent affinities for cocaine, GBR12909, or β-CIT. In contrast, a recent study using DAT/NET chimeras found that replacing the DAT N terminus with that of NET enhanced the DAT affinity for DA, whereas replacing the NET N terminus with that of DAT reduced NET affinity for DA (18). Consistent with our results, they also observed that C-terminal exchanges had no effect on DA affinity. Further, a study from Vaughan and co-workers (42) found that N-terminal point mutations (S4A, C6A, S7A, and S7D) in rat DAT exhibited reduced β-CFT bind-
However, although cocaine, DAT N-terminal substitution. A previous report identified a halide, iodide, in diverse compounds (see chemical structures; Fig. 3, observed that this occurred over a broad range of structurally stability of the ligand-bound conformation. Interestingly, we with a model in which the DAT N terminus may influence the affinity of select inhibitors.

ing, further supporting that the DAT N terminus contributes to the affinity of select inhibitors.

Taken together with our findings, these data are consistent with a model in which the DAT N terminus may influence the stability of the ligand-bound conformation. Interestingly, we observed that this occurred over a broad range of structurally diverse compounds (see chemical structures; Fig. 3, B and C). However, although cocaine, β-CFT, and β-CIT are all tropane derivatives, only β-CFT apparent affinity was affected by the DAT N-terminal substitution. A previous report identified a DAT point mutation (D345N) that is insensitive to β-CFT but retains sensitivity to cocaine inhibition (43), suggesting that binding of tropanes derivatives within DAT is not identical. Moreover, a dDAT crystal structure encoding point mutations that mimic hDAT displayed enhanced cocaine and β-CFT, but not β-CIT, binding (38), consistent with a model in which these compounds have differential binding properties for DAT. Cocaine and β-CIT are larger molecules than β-CFT. Cocaine’s ester linkage (absent in β-CIT and β-CFT) increases its size compared with β-CFT, whereas the substitution of the larger halide, iodide, in β-CIT increases its size compared with β-CFT. This raises the possibility that the molecular size of these compounds exerts a large influence on their ability to bind within the S1 binding pocket. Consistent with this premise, DA, AMPH, and MPH, which are all small DA-related compounds, were all sensitive to the N-terminal substitution, whereas GBR12909, which is a large, extended piperazine derivative, was not impacted by the DAT N-terminal substitution.

The potential explanation for the observed different shifts in inhibitory potency is an energy change for reaching the inhibitor-transporter complex state, which does not affect every inhibitor in the same way. Thus, the intracellular termini substitution may have caused structural changes in the outer vestibule or changed the free energy hypersurface of the translocation process. Most inhibitors bind competitively to DAT and prevent the transition from outward facing to inward facing. It is conceivable that larger compounds interfere already in the initial part of the transition, whereas smaller inhibitors block the transporter movement along the transition path. The geometry of the outward facing conformation is most likely unchanged, because the $K_i$ of the largest compounds did not change. More intensive structural and/or computational interrogations will be necessary to discern among these possibilities.

How does the N terminus influence the coordination of substrates and inhibitors in the DAT binding pocket? The adaptation of DAT to molecule size within its pocket was demonstrated by Gouaux and co-workers (38) using the same dDAT crystal structures bound to substrates and inhibitors. In their study, they posit that the small substrates sit precisely within the pocket in appropriate position. The larger inhibitors, like tropanes and antidepressants, lock DAT in an outward facing conformation to exert their effects. A notable difference between their findings and ours is that, as stated previously, they do not find any difference between inhibitor binding sites for cocaine and β-CFT; however, their study focused on dDAT, whereas ours investigated hDAT. Although the binding sites of hDAT and dDAT share ~80% identity, dDAT exhibits 10-fold lower affinity for cocaine than hDAT (44). Another potential difference between our findings and the previous results is that although their constructs bind DA and inhibitors, they lack DA uptake function. The dDAT crystal structures omit much of extracellular loop 2 and the N terminus. Complete N-terminal truncation (residues 1–66) results in a loss of DA uptake (11), truncation of residues 1–60 results in marked loss of transporter uptake velocity (45), and a previous DAT truncation mutant lacking residues 1–59 exhibits some measurable uptake, although neither the kinetic constants nor expression levels were reported for Δ1–59 DAT mutant (46). Work from our laboratory likewise suggests that truncations within the N terminus significantly impair transporter expression and function (data not shown). Our current study has the benefit of using N- and C-terminal substitutions on an otherwise complete WT background, such that DA uptake could be assessed in robustly expressing transporters.

Another factor that may contribute to role of the DAT N terminus in substrate/inhibitor affinities is the interaction of the DAT N terminus with PIP$_2$ and intracellular loop 4 (34, 47).
A recent molecular dynamics study examining the hDAT N terminus on the dDAT background reported a PIP$_2$-mediated interaction with hDAT N-terminal residues (Lys$^5$, Lys$^6$, and Arg$^{31}$) with intracellular loop 4 (34). This interaction appears to break salt bridges necessary for the conserved intracellular gate. These findings contrast with simulation studies examining hSERT, where the N terminus moves further away from intracellular domains following transition to the inward-open state (35). It should be noted that PIP$_2$ binds to SERT at its intracellular loops (48), but whether PIP$_2$ affects SERT N-terminal mobility remains untested. We did not detect differences in substrate/inhibitor apparent affinities among the SERT core chimeras. The reported differential trajectories for DAT and SERT N-terminal domains during transition to the inward-open state may result in differential effects on substrate/inhibitor binding sites. These opposing trajectories may explain why we see differences in effects between DAT core and SERT core chimeras.

In summary, we have identified a putative role for the DAT N terminus in influencing the substrate/inhibitor binding site, and a possible synergistic role for the DAT N and C termini to coordinate accessibility to the binding site. These findings will provide critical information to future structural studies that incorporate DAT intracellular domains in structural analyses.

**Experimental Procedures**

**Materials**—Rat anti-DAT (MAB369) antibody was from Millipore, and mouse anti-SERT antibody was from mAb Technologies. HRG-conjugated anti-rat secondary and mouse anti-actin (SPM161) antibodies were from Santa Cruz, and HRP-conjugated anti-mouse antibody was from Jackson Immunoresearch Laboratories (115-035-003). Sulfo-NHS-SS-biotin and streptavidin agarose were from Thermo Scientific. Immunoresearch Laboratories (115-035-003). Sulfo-NHS-SS-biotin and streptavidin agarose were from Thermo Scientific. HRP-conjugated anti-rat secondary and mouse anti-actin (SPM161) antibodies were from Santa Cruz, and HRP-conjugated anti-mouse antibody was from Jackson Immunoresearch Laboratories (115-035-003). Sulfo-NHS-SS-biotin and streptavidin agarose were from Thermo Scientific. HRP-conjugated anti-rat secondary and mouse anti-actin (SPM161) antibodies were from Santa Cruz, and HRP-conjugated anti-mouse antibody was from Jackson Immunoresearch Laboratories (115-035-003).

**cDNA Constructs**—Chimeric hDAT and hSERT cDNAs were generated using the PCR-ligation-PCR approach (36), using hDAT pcDNA3.1 (+) and/or hSERT pcDNA3.1 (+) as templates, corresponding to the following coding regions: N-SERT/DAT (hSERT[1–78]/hDAT[60–620]), DAT/C-SERT (hDAT[1–583]/hSERT[601–630]), SERT/DAT/SERT (hSERT[1–78]/hDAT[60–583]/hSERT[601–630]), N-DAT/SERT (hDAT[1–59]/hSERT[79–630]), SERT/C-DAT (hSERT[1–600]/hDAT[584–620]), and SERT/DAT/SERT/DAT (hDAT[1–59]/hSERT[79–600]/hDAT[584–620]). The resulting chimeric cDNAs were digested and cloned back into their parental transporters at the following restriction sites: HindIII/PflMI (N-SERT/DAT, SERT/DAT/SERT), HindIII/AgeI (N-DAT/SERT, DAT/SERT/DAT), or Clal/XbaI (DAT/C-SERT, SERT/C-DAT, SERT/DAT/SERT). All chimeric constructs were confirmed by Sanger sequencing (Genewiz).

**Cell Culture and Generation of Pooled Stable Cell Lines**—Human dopaminergic SK-N-MC cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained in MEM supplemented with 10% fetal bovine calf serum, 100 units/ml penicillin/streptomycin, 2 mM l-glutamine, 37 °C, 5% CO$_2$. The cells were transfected with wild type or chimera cDNAs using Lipofectamine 2000 according to the manufacturer’s instructions with the following modifications: the cells were seeded into 6-well plates at a density of $1 \times 10^5$ 1 day prior to transfection and were transfected with 3 μg of plasmid DNA/well using a lipoid:DNA ratio of 2:1. To generate stable cell lines, 48 h following transfection, the cells were selected using 0.5 mg/ml G418. Stably transfected cells were pooled, and cell lines were maintained under selective pressure using 0.2 mg/ml G418.

**Uptake Assays**—[3H]DA and [3H]5HT uptake: 7.5 $\times$ 10$^4$ (DA uptake) or 5.0 $\times$ 10$^4$ (5HT uptake) cells/well were seeded onto 96-well plates, 1 day prior to the assay. The cells were washed twice with room temperature Krebs–Ringers–HEPES buffer (KRH: 120 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 2.2 mM CaCl$_2$, 10 mM HEPES, pH 7.4), and transport assays were conducted as described below. All transport reactions proceeded for 10 min at 37 °C and were terminated by washing thrice with ice-cold KRH buffer. The cells were solubilized in scintillation fluid for 15 min at room temperature, and [3H] accumulation was quantified using a Wallac Microbeta scintillation plate counter (PerkinElmer Life Sciences). Triplicate points were measured for each data point, and non-specific uptake was defined in the presence of either 10 μM GBR12909 (DA uptake) or 10 μM paroxetine (5HT uptake). The data were analyzed using Excel and GraphPad Prism software. Mixture solutions were counted in parallel, and accumulated [3H] was less than 10% of the total [3H] added for all assays.

**Kinetics**—[3H]DA (3.4–[ring-2,5,6,6-3H]dihydroxyphenylethylamine; Perkin Elmer) and [3H]5HT ([3H]-5-hydroxytryptamine; PerkinElmer) 10× cocktails were prepared by diluting a 1/50th volume radiolabeled substrate into 60 μM stock solutions of unlabeled DA or 5HT in KRH containing 0.18% glucose and 10 μM each pargline and sodium ascorbate (KRH/g/p/a). 60 μM [3H]substrate solutions were serially diluted into KRH/g/p/a to generate [3H]DA or [3H]5HT cocktails at 10× the indicated final substrate concentrations. The cells were preincubated (20 min, 37 °C) with KRH buffer supplemented with 0.18% glucose (KRH/g), and uptake was initiated by adding a 1/10th volume of 10× concentrated [3H] substrate cocktails.

**Inhibitor Dose-Response Curves**—The cells were preincubated in KRH/g (30 min, 37 °C) with the indicated drugs at the indicated concentrations. Uptake was initiated by adding a 1/10th volume of 500 nM [3H]DA or 250 nM [3H]5HT 10× mixture, for final assay concentrations of 50 nM (DA) or 25 nM (5HT).

**AMPH and Fenfluramine Dose Response**—The cells were preincubated in KRH/glucose buffer (20 min, 37 °C). Uptake was initiated by adding a 1/10th volume of either 500 nM [3H]DA or 250 nM [3H]5HT 10× mixture, containing AMPH or fenfluramine, respectively, at 10× the indicated final concentrations.

**Cell Surface Biotinylation**—Surface proteins were covalently labeled with sulfo-NHS-SS-biotin as previously described (12, 37). Briefly, the cells were rapidly chilled by washing in ice-cold PBS, pH 7.4, supplemented with 1.0 mM MgCl$_2$, 0.1 mM CaCl$_2$ (PBS$^{37}$), and incubated with 1.0 mg/ml sulfo-NHS-SS-biotin in ice-cold PBS$^{37}$, twice, for 15 min at 4 °C. Residual reactive NHS groups were quenched by repeated washing and incubating.
twice in PBS2+/100 mm glycine for 15 min at 4 °C. The cells were washed three times in ice-cold PBS2+ and lysed in radioimmune precipitation assay buffer containing protease inhibitors, and protein concentrations were determined using the BCA protein assay (Pierce). Biotinylated proteins from equivalent amounts of cellular protein were isolated by batch streptavidin chromatography (overnight, 4 °C), and bound proteins were eluted in 2× SDS-PAGE sample buffer for 30 min at room temperature. Either supernatants (WT DAT, DAT/C-SERT) or total cellular lysates (N-SERT/DAT, SERT/DAT/SERT) were used to normalize surface levels. The blots were probed with actin for normalization (N-SERT/DAT, SERT/DAT/SERT). The samples were resolved by SDS-PAGE, and specific proteins were detected by immunoblotting with the indicated antibodies. Immunoreactive bands were detected with SuperSignal West Dura (Pierce) and were captured using a VersaDoc Imaging station (Bio-Rad). Non-saturating bands were quantified using Quantity One software (Bio-Rad). Relative surface expression was quantified as percentage of surface protein (WT DAT, DAT/C-SERT, N-DAT/SERT, DAT/SERT/DAT). The data were normalized to percent WT DAT (DAT/C-SERT), N-SERT/DAT (SERT/DAT/SERT), or N-DAT/SERT (DAT/ SERT/DAT).  

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