The Dopamine Transporter Carboxyl-terminal Tail

TRUNCATION/SUBSTITUTION MUTANTS SELECTIVELY CONFER HIGH AFFINITY DOPAMINE UPTAKE WHILE ATTENUATING RECOGNITION OF THE LIGAND BINDING DOMAIN*

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In order to delineate structural motifs regulating substrate affinity and recognition for the human dopamine transporter (DAT), we assessed [3H]dopamine uptake kinetics and [3H]CFT binding characteristics of COS-7 cells transiently expressing mutant DATs in which the COOH terminus was truncated or substituted. Complete truncation of the carboxyl tail from Ser582 allowed for the expression of biphasic [3H]dopamine uptake kinetics displaying both a low capacity (Vmax~0.4 pmol/10⁶ cells/min) high affinity (Km~300 nM) component and one exhibiting low affinity (Km~15 μM) and high capacity (Vmax~5 pmol/10⁶ cells/min) with a concomitant 40% decrease in overall apparent Vmax relative to wild type (WT) DAT. Truncation of the last 22 amino acids or substitution of the DAT-COOH tail with sequences encoding the intracellular carboxyl-terminal of either dopamine D1 or D5 receptors produced results that were identical to those with the fully truncated DAT, suggesting that the induction of biphasic dopamine uptake kinetics is likely conferred by removal of DAT-specific sequence motifs distal to Pro597. The attenuation of WT transport activity, either by lowering levels of DAT expression or by pretreatment of cells with phorbol 12-myristate 13-acetate (1 μM), did not affect the kinetics of [3H]dopamine transport. The estimated affinity of dopamine (Kd~180 nM) for all truncated/substituted DAT mutants was 10-fold lower than that of WT DAT (~2000 nM) and appears selective for the endogenous substrate, since the estimated inhibitory constants for numerous putative substrates or uptake inhibitors were virtually identical to those obtained for WT DATs. In marked contrast, DAT truncation/substitution mutants displayed significantly reduced high affinity [3H]CFT binding interactions with estimated Kd values for dopamine and numerous other substrates and inhibitors tested from 10-100-fold lower than that observed for WT DAT. Moreover, co-expression of truncated and/or substituted DATs with WT transporter failed to reconstitute functional or pharmacological activities associated with both transporters. Instead, complete restoration of uniphasic low affinity [3H]dopamine uptake kinetics (Km~2000 nM) and high affinity substrate and inhibitor [3H]CFT binding interactions attributable to WT DATs were evident. These data clearly suggest the functional independence and differential regulation of the dopamine translocation process from the characteristics exhibited by its ligand binding domain. The lack of functional phenotypic expression of mutant DAT activities in cells co-expressing WT transporter is consistent with the contention that native DATs may exist as multisubunit complexes, the formation and maintenance of which is dependent upon sequences encoded within the carboxyl tail.

The Na⁺- and Cl⁻-dependent nigral-striatal presynaptic dopamine transporter (DAT), by mediating the reuptake of dopamine into the cell, significantly regulates the availability of synaptic dopamine (DA) to effectively interact with multiple pre- and postsynaptic dopamine receptors (for reviews see Refs. 1–4). The DAT is the presumed major target site for the accumulation of the dopaminergic neurotoxin, 1-methyl-phenylpyridine (MPP⁺), producing Parkinson's like symptoms (5), and the initial site of action of psychostimulants and drugs of abuse, such as amphetamine, methylphenidate, and cocaine to elicit psychomotor behavior associated with euphoria, self-reward, and addiction (see Refs. 6–10). The isolation of cDNA clones for the mouse (11), rat (12–14), bovine (15) and human DATs (16–19) has revealed that it is a member of a large gene family of Na⁺- and Cl⁻-dependent transporters (20–22) with particular homology to neurotransmitter transport proteins for noradrenaline (NE) (23) and serotonin (24). As with other members of the family, hydrophatic analysis and molecular modeling (25) of the amino acid sequence of the DAT predicts a common topology of 12 putative transmembrane (TM) domains, a large extracellular loop between TM 3 and 4 containing numerous consensus sequences for N-linked glycosylation, and potential sites for phosphorylation by protein kinase A, protein kinase C, and CaM kinase II within putative intracellular domains and in the amino and carboxyl termini. The human and rat DATs are encoded by polypeptides of ~69 kDa with 92% overall amino acid sequence homology. Upon heterologous expression in mammalian cells, the doped DAT confers Na⁺- and Cl⁻-dependent bidirectional dopamine transport (18, 26) and cytotoxicity to MPP⁺ (27, 28), which is sensitive to the inhibition by DAT antagonists including cocaine, methylpheni-
date, and mazindol with an expressed rank order of potency similar to that observed in native brain synaptosomes. Similarly, there is a strong correlation between the relative potencies of various substrates and inhibitors of \(^{[3]H}\) dopamine uptake with those potencies or \(K_i\) values with which these compounds inhibit binding of radiolabeled ligands, such as \(^{[3]H}\) CFT and \(^{[3]H}\) IBBR-12935 to either native neuronal (29, 30) or cloned human DATs (19, 31).

Knowledge of DAT primary sequence has allowed for the identification of specific regions and amino acid residues that regulate various transport functions. Studies employing site-directed mutagenesis (32) and chimeric dopamine-noradrenaline transporters (33–35) have attempted to identify and delineate discrete functional domains responsible for substrate and inhibitor interactions with DAT. Based on this work it appears that amino acids in regions encoding TM 1–3 and 9–11 are important in defining substrate affinity, while sequence motifs spanning TM 5–8 are responsible for inhibitor interactions and translocation efficiency (34). Although evidence from both biochemical (30, 36–38) and behavioral (9, 39–42) studies support the dissociation of the ligand binding domain from the dopamine uptake process, the relationship and exact nature of these events has still yet to be clearly defined.

While significant advances have been made in the gross molecular dissection of DAT structure-function relationships, little information is available regarding the functional role of putative intracellular loops and, particularly, domains within either the amino or carboxyl terminus. While the COOH-tail appears necessary for correct processing and trafficking of some Na\(^+\)-dependent transport proteins to appropriate membrane compartments (43–46), both amino and carboxyl-terminal domains appear not to be required for GABA transport (47, 48), and swelling of the COOH-tail of the serotonin transporter with corresponding sequence from the NE transporter displayed no apparent effect on serotonin uptake/activity (49). In contrast, the region encompassing TM 12 to the COOH-terminal tail of the serotonin (50) and of distantly related glucose (51, 52) and galactose (53) transporters may be important determinants for substrate/inhibitor recognition.

In order to further delineate the structural motifs that may regulate dopamine uptake affinity and its relationship to the ligand binding domain, we assessed the \(^{[3]H}\) dopamine uptake kinetics and \(^{[3]H}\) CFT ligand binding characteristics of hDAT mutants in which the COOH-terminal tail was completely/partially truncated or replaced by unrelated sequences encoding the COOH tail of dopamine D1/D5 receptors. We provide evidence for the functional independence of the dopamine translocation process from the characteristics exhibited by its ligand binding domain and evidence that removal of sequences encoded within the last 22 amino acids of the COOH-terminal tail allows for the expression of substrate uptake affinity mimicking that seen in native tissues. Moreover, we hypothesize that the expression of high affinity ligand binding interactions and low affinity dopamine uptake kinetics in cell lines expressing the cloned hDAT is possibly mediated by DAT multisubunit complexes, the formation and maintenance of which is dependent upon sequences encoded within the carboxyl tail.

**EXPERIMENTAL PROCEDURES**

Construction of hDAT Mutants—A full-length human DAT cDNA, previously isolated from a human substantia nigra library (19), was utilized as a template in PCR in combination with specific oligonucleotide primers to create complete and partial carboxyl-terminal truncated hDAT mutants termed hDAT-tr1 and hDAT-tr2, respectively (see Fig. 1). Three primers were synthesized (Biotechnology Service Centre, Hospital for Sick Children, Toronto): A1, 5'-CCCTGCCATTACACCTCCAGTGTTGCCCAT-3'; A2, 5'-CAGAGATTCTTATGCCTGCAAAGGTAGGG-3'; A3, 5'-CTCGAATTCTTAGGGTGCAATTGGCCTAGGC-3'.

Primer A1 encodes nucleotides 1–22 immediately 5' to the putative initiation codon of the EcoRI hDAT (19) and incorporates a KpnI restriction site (boldface type). A2 and A3 primers encode antisense nucleotides 1769–1748 and 1813–1799 of hDAT cDNA (48), Ala\(^{577}\)-Ser\(^{582}\) and Ala\(^{595}\)-Pro\(^{597}\), respectively, both of which incorporate an artificial stop codon (underlined) in addition to an EcoRI restriction site (boldface type) to facilitate subsequent subcloning. The full truncation mutant was created by subjecting hDAT cDNA to PCR amplification with A1 and A2 primers, while DAT-tr2 construct was obtained following amplification with A1 and A3 primers. Approximately 200 ng of the PCR product was subjected to 30 cycles of PCR (Perkin-Elmer) with 0.5 \(\mu\)g of primer A1 and appropriate synthetic oligodeoxynucleotide (A2 or A3) containing 2.5 units of Taq polymerase and 200 \(\mu\)M dNTPs under the following conditions: 1 min at 95°C for denaturation; 1 min at 60°C for primer annealing, and 1.5 min at 72°C for primer extension. Amplified products (20 \(\mu\)l) were electrophoresed, and extracted DNA was subjected to phenol-chloroform extraction and ethanol precipitation.

Dopamine D1 and D5 receptor intracellular carboxyl tails encoding amino acids 337–446 and 362–477, respectively, were amplified by PCR using the following primers: B1, 5'TTCTCCTGCAACGCGGAGCTGCAGTGTGCCCAT-3'; B2, 5'TTCTCCTGCAACGCGGAGCTGCAGTGTGCCCAT-3'; B3, 5'TTCTCCTGCAACGCGGAGCTGCAGTGTGCCCAT-3'; B4, 5'TTCTCCTGCAACGCGGAGCTGCAGTGTGCCCAT-3'. Primers B1 and B3 both contain a PstI restriction site (boldface type) and encode nucleotides 1081–1100 and 997–1016 immediately 3' to TM 7 of dopamine D5 and D1 receptors, respectively. Both B2 and B4 primers incorporate a Sp6 restriction site (boldface type) and are complementary to the sequence encoding nucleotides 1457–1437 and 1360–1340, of the 3'-untranslated region of the dopamine D5 and D1 receptors, respectively. These primers were utilized in PCR with ~200 ng of human dopamine D1 (54) and D5 (55) receptor DNA to amplify dopamine receptor COOH tail DNA fragments (B1 and B2 for the D5 receptor, B3 and B4 for the D1 receptor) under conditions described above. Digestion of hDAT tail DNA with PstI (3' to putative TM 12) produced a COOH tail-truncated hDAT-fragment (Cy5\(^{540}\)) that was ligated to PstI-digested amplified dopamine D1 or D5 receptor DNA and subcloned into EcoRI- and Sp6-digested pcDNA3. To confirm the absence of spurious PCR-generated nucleotide errors, appropriate construction of splice fusion, and stop codon insertions, all hDAT mutants were sequenced in both directions using 7-deaza-dGTP and Sequenase, version 2.0 (U.S. Biological Corp.) with specific internal primers as described previously (19).

**Cell Transfection and Assay—**For transient expression studies, COS-7 cells, cultured in Dulbecco's α-modified Eagle's medium supplemented with 10% fetal calf serum and 5% CO\(_2\) at 37°C, were transfected with cesium-purified expression vector pcDNA encoding either WT or a mutant DAT cDNA by the calcium phosphate precipitation method (4–40 \(\times\) 10\(^5\) cells; 48 ohms, 135 mA, 500 microfarads) as described previously (19).

**[^3]H]Dopamine Uptake—**Measurement of dopamine uptake was performed on intact cells essentially as described (19). Briefly, 2–4 days following transfection in 24-well plates (2–10 \(\times\) 10\(^4\) cells seeded per well) medium was removed, and wells were rinsed with 0.5 ml of uptake buffer containing 5 mn Tris, 7.5 mM D-glucose, 120 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 1 mM ascorbic acid, 5 mM glucose, pH 7.1. Cells were reincubated in duplicate with the indicated concentrations of dopaminergic agents (10\(^{-10}\) to 10\(^{-4}\) \(\mu\)M) 5 min prior to the addition of 0.25 ml of uptake buffer containing 20 nm [\(^{3}H\)]DA (final concentration) or [\(^{3}H\)]noradrenaline (40 nm final concentration where indicated) and incubated for 10 min at room temperature in a total volume of 0.5 ml. Nonspecific [\(^{3}H\)]dopamine (37–53 Ci/mmol) uptake was measured in the presence of 10 \(\mu\)M mazindol. Wells were rinsed twice with 0.5 ml of uptake buffer, and cells were solubilized in 1% SDS (0.5 ml) for 15 min at 22°C with incorporated radioactivity measured by liquid scintillation spectrometry in a Beckman LS 6000SC.

**[^3]H]CFT Binding—**[^3]H]CFT binding to COS-7 cells transfected with WT or mutant human DAT was measured as described previously (19) under conditions similar to those defined above. Briefly, medium was removed, and cells were rinsed with 0.5 ml of uptake buffer. For saturation ([\(^{3}H\)]CFT (82–85 Ci/mmol, Du Pont NEN) binding experiments, cells were incubated in duplicate with 0.25 ml of ice-cold uptake buffer containing increasing concentrations of [\(^{3}H\)]CFT (0.125–200 nm, final concentration) in a total volume of 0.5 ml. Following incubation for 2–3 h at 4°C, wells were washed twice with 0.5 ml of ice-cold buffer, and cells were solubilized in 1% SDS with bound radioligand measured by liquid scintillation counting as described above. For [\(^{3}H\)]CFT competition binding experiments, cells were incubated in duplicate with 0.25 ml of ice-cold uptake buffer containing [\(^{3}H\)]CFT (4–10 nm final concentra-
38 amino acid residues (583–620) were removed (see Fig. 1) (Ser582), termed hDAT-tr1, was constructed in which the last transport function, an hDAT COOH tail truncation mutant COOH-terminal tail may exert on the expression of dopamine 10-fold concentration range of DNA studied, functional DAT activity cells) of WT pCD DAT vector DNA used for cell transfection. Over the concentration) and 0.25 ml of competing ligand (10⁻⁷ M mazindol). Assays were conducted in parallel, using the same serial dilution of drug, and on the same batch of transfected cells.

Regulation of DAT Levels—In experiments assessing the role of DAT expression levels on [³H]dopamine uptake kinetics, two methods were used to reduce levels of DAT activity. Reductions of functional uptake levels by up to ~50% was achieved by pretreating cells with the protein kinase C activator, PMA (56). COS-7 cells transiently transfected with either WT or mutant pCD-hDATs were washed once with uptake buffer followed by the addition of 0.5 ml of 1 M PMA (final concentration) in uptake buffer and incubated for 1 h at room temperature. Cells were washed once in uptake buffer and assayed for [³H]DA uptake kinetics as described above. Reductions of DAT protein levels were achieved by lowering the concentration range (from 40 to 4 µg of DNA/2.5 × 10⁶ cells) of WT pCD DAT vector DNA used for cell transfection. Over the 10-fold concentration range of DNA studied, functional DAT activity was reduced by up to ~60%. Three to four days following transfection, cell pellets were processed for [³H] DA uptake and [³H]JCF binding as described above.

Co-expression Studies—For co-transfection experiments, COS-7 cells were initially transfected by electroporation with WT and DAT truncation/substitution mutants, using the same amount of DNA (~40 µg) for each construct. In order to produce co-expression of WT and subtype-specific hDAT mutants essentially displaying equivalent level of functional uptake, COS-7 cells were transfected with 4 and 40 µg of DNA of WT DAT and mutant DAT, respectively, and assayed simultaneously for either [³H]DA uptake or [³H]JCF binding as described above. Control cells were transfected with ~30 µg of pcDNA vector and 4 µg of WT DAT or with ~40 µg of each of the mutant DATs alone. Verification of DAT protein co-expression and lack of possible homologous recombination events were assessed by immunoblotting as described below.

Immunoblot Analysis—COS-7 cells transiently transfected with 4 µg of WT DAT and 40 µg of DAT-D1 recombinant plasmid DNA were collected and homogenized using a Polytron (Brinkman) in 5 mm Tris buffer, pH 7.4, containing protease inhibitors (2 mM EDTA, 10 µg/ml benzamid, 5 µg/ml leupeptin, 5 µg/ml soybean trypsin inhibitor) and spun at 4°C for 30 min at 48,000 × g. The pellet was solubilized in sample buffer and recentrifuged as above. The resultant pellet was solubilized in sample buffer containing 10% SDS and 1% β-mercaptoethanol at room temperature for 1 h. Aliquots of solubilized protein (~40 µg) were dot-blotted onto polyvinylidene difluoride membranes (Millipore Corp.) and blocked with 20 ml Tris-buffered saline, pH 7.6, containing 1.1% Tween 20 (IBS-T) and 5% skim milk for 1 h at room temperature. Blots were subsequently incubated overnight at 4°C with either primary DAT or human D1 polyclonal antibodies as described previously (57, 58) at a dilution of 1:12,000. Blots were extensively washed and incubated for 1 h at room temperature with peroxidase-conjugated donkey anti-rabbit IgG essentially as described by the manufacturer (Amersham Corp.). Immunoreactivity was detected by enhanced chemiluminescence on XAR film (Kodak). Human hDAT antibodies recognize epitopes within the amino-terminal tail and large extracellular loop of DAT (57), while D1 receptor antibodies recognize epitopes within the COOH tail of the human D1 receptor (58). The use of DAT and dopamine D1 receptor antibodies in cells co-expressing DAT and D1-D5 (an epitope-tagged functional DAT mutant) allowed the documentation of membrane co-expression of the two proteins.

Data Analysis—Estimated Kₘ and Vₘₐₓ values for [³H]DA uptake and Kₘ and Bₘₚ values for [³H]JCF binding data were analyzed using nonlinear least-squares curve-fitting program KALEIDAGRAPH (Abelbeck Software), LIGAND, and/or GraphPad PRISM (GraphPad Software Inc.) as described previously (19). Simultaneous statistical analysis of a one-site versus two-site model were made using GraphPad PRISM or LIGAND in which the two-site model was preferred only when the F-test displayed significance at the p < 0.01 level.

RESULTS AND DISCUSSION

In order to investigate the possible regulatory effects the COOH-terminal tail may exert on the expression of dopamine transport function, an hDAT COOH tail truncation mutant (Ser582), termed hDAT-tr1, was constructed in which the last 38 amino acid residues (583–620) were removed (see Fig. 1) and assayed for its ability to mediate [³H]DA transport activ-

Fig. 1. Schematic illustration of mutant DAT constructs. Shown is a map of native DAT depicting putative TM domains, the intracellular location of both the amino and carboxyl termini, and the location of either the full (hDAT-tr1) or partially truncated (hDAT-tr2) carboxyl tail DAT mutants. Also depicted are hDAT-chimeric constructs in which hDAT tail sequence was substituted by amino acid residues encoding the COOH-terminal tail of dopamine D1 (hDAT-D1) or D5 (hDAT-D5) receptors. Although hDAT-tr1 and the hDAT-D5 mutant lost all putative COOH-terminal phosphorylation sites, hDAT-tr2 retained consensus sequence for protein kinase C sites while the hDAT-D1 mutant contains only one functional protein kinase A site (85).

To assess whether the altered dopamine transport kinetics of hDAT-tr1 were due to the specific removal of DAT carboxyl-terminal amino acid residues and not merely the product of altered DAT net charge or overall length, two additional hDAT mutants, termed hDAT-D1 and hDAT-D5, were constructed in which the hDAT CT tail was removed and substituted with totally unrelated amino acid sequence motifs encoding the putative intracellular carboxyl-terminal domains of either dopamine D1 (54) or D5 receptors (55) (amino acid residues 337–446 for D1 and 362–477 for D5). The addition of dopamine D1 and D5 receptor COOH tail sequence effectively added over 60 amino acids to the total length of the denatured hDAT, changed its net charge by up to ~16, and added structural domains putatively involved in receptor desensitization and palmitoylation (59). Both substitution mutants exhibited saturable [³H]DA uptake kinetics identical to hDAT-tr1, revealing biphasic DA uptake characteristics with a high affinity (Kₘ ~ 300 nM) low capacity (Vₘₚ ~ 0.3 pmol/10⁶ cells/min) component and a low affinity (Kₘ ~ 15 µM) high capacity (Vₘₚ ~ 5 pmol/10⁶ cells/min) component. Estimated Kₘ and Vₘₚ values for WT and hDAT
mutants are listed in Table I. Similar to hDAT-tr1, both hDAT-D1 and hDAT-D5 mutants displayed [3H]dopamine uptake levels that were reduced by up to 50% relative to WT hDAT (Table I). Thus, despite the lack of appreciable homology between dopamine D1 and D5 carboxyl tail sequences and the presence of distinct post-translational motifs, both mutants exhibited reduced [3H]dopa uptake kinetics that were not only identical to each other but also virtually identical to the truncation mutant, hDAT-tr1. As such, the apparent regulation of DA uptake kinetics by hDAT COOH tail appears to be due to sequences specific to hDAT.

To further define which carboxyl-terminal amino acid residues may allow for the expression of biphasic uptake kinetics and the observed increase in high affinity [3H]dopamine uptake, an hDAT mutant, termed hDAT-tr2, was constructed in which the last 23 amino acids (598–620) of the COOH tail were removed. As shown in Table I, hDAT-tr2-mediated [3H]dopamine uptake kinetics were virtually identical to hDAT-tr1 and to both hDAT-D1 and hDAT-D5, displaying biphasic saturable DA uptake components with expressed high and low affinity, and were similarly accompanied by an observable ~40% loss of [3H]dopamine uptake compared with WT hDATs.

While overall loss of detectable transport activity can possibly be accounted for by truncation-induced aberrations in cell surface trafficking or protein stability (61), the observed 40–50% reduction in the functional expression level of all hDAT mutants does not, however, contribute to either the observed biphasic [3H]dopamine uptake kinetics or unmasking of a saturable high affinity transport component. Thus, functionally reducing WT hDAT DA uptake activity to a level comparable with that of hDAT truncation mutants, either by lowering protein expression (from 9.0 to 3.0 pmol/10^6 cells/min, n = 8) or by reducing functional uptake by PMA pretreatment (from 7.0 to 5.0 pmol/10^6 cells/min, n = 4), as described under “Experimental Procedures,” did not significantly influence estimates of DA uptake affinity (K_m values ranged from 1.8 to 2.4 μM, n = 4) and did not induce biphasic uptake kinetics for DA under any of the conditions tested (data not shown). The observed selective effects of PMA on [3H]dopamine uptake velocity are identical to those described in a previously published report (56). All hDAT mutants appeared more sensitive to PMA treatment, however, with functional [3H]dopamine uptake reductions of up to 80%. While accurate determinations of [3H]dopamine uptake kinetics of hDAT mutants were difficult to obtain under these conditions, the estimated K_m of dopamine transport for remaining PMA-resistant transport sites remained unchanged (~150–200 nM, n = 3; data not shown). Moreover, although EC_{50} values were not determined, the observed enhancement in dopamine uptake affinity by all hDAT mutants does not appear to be attributable to altered Na^+ transport requirements, since replacement of Na^+ ions from the uptake medium by Li^+, as described (62), virtually abolished [3H]dopamine uptake activity of both WT and all hDAT mutants (data not shown, n = 2) and corroborates the notion that COOH tail regions are not primarily involved in the ionic dependence of the DA uptake process (33, 34). Furthermore, all hDAT mutants displayed initial rates of [3H]dopamine uptake similar to WT hDAT, remaining linear for up to 20 min (n = 2; data not shown). Taken together, the data lessen the possibility that lowered DA uptake levels, modified sensitivity to PMA, or ionic requirements contribute to the observed altered kinetics and affinity for DA transport by these hDAT mutants.
As illustrated in Fig. 2B, under conditions of low substrate concentrations (20 nM [3H]DA), hDAT-tr1-mediated uptake was inhibited in a concentration-dependent manner by unlabeled DA with an estimated inhibitory constant (K_i, ~170 nM) 10-fold lower than that exhibited (K_i, ~1.6 μM) by WT hDAT and similar to the observed K_m values in native brain synaptosomes. Moreover, as depicted in Fig. 2B and as described above, all hDAT mutants, following expression in COS-7 cells, exhibited [3H]DA uptake that was competitively inhibited by dopamine in a concentration-dependent manner with observed estimated inhibitory constants of ~200 nM, virtually identical to that observed for hDAT-tr1, and of 10-fold higher affinity than WT hDAT. As seen from the data presented in Table I, the estimated proportion of high affinity [3H]DA uptake sites exhibited by these hDAT mutants represents a small proportion (~10%) of the total V_max. Whereas these values may be somewhat underestimated by the kinetic models used here (see Refs. 53 and 60), it appears that the observed 10-fold increase in estimated affinity for dopamine uptake exhibited by hDAT-tr1, hDAT-D1, hDAT-D5, and hDAT-tr-2 (Fig. 2B) under conditions of low substrate concentrations is attributable to only one of two distinct saturable uptake processes.

To investigate the specificity of truncating or substituting the hDAT COOH tail on DA uptake affinity, several different compounds from numerous structural classes, including uptake inhibitors and substrates, were examined for their ability to inhibit [3H]DA uptake. As illustrated in Fig. 3A, [3H]DA-mediated uptake by hDAT-tr1 was inhibited by substrates, such as amphetamine and noradrenaline, in a concentration-dependent and uniphasic manner with displayed IC_{50} values that were virtually identical to those of WT hDAT. Similarly, as depicted in Fig. 3B, dopamine transport inhibitors, such as GBR-12909 and bupropion, did not appear to differentiate between the WT and mutant hDAT-tr1, exhibiting IC_{50} values for [3H]DA uptake similar to WT hDAT controls. Virtually identical results were obtained for all other hDAT mutants tested. Estimated K_i values for these and numerous other putative substrates and inhibitors of dopamine transport mediated by hDAT COOH tail mutants are listed in Table II. As is evident from the data presented, while some compounds displayed K_i values for [3H]DA uptake that varied by 2–3-fold among the various transporter mutants, all compounds exhibited a rank order of potency and pharmacological profile clearly indicative of hDAT activity. Only dopamine displayed estimated K_i values for [3H]DA uptake that were significantly and consistently at least 10-fold lower for all hDAT mutants compared with WT hDAT. The selectivity of COOH-tail truncation on dopamine uptake affinity and catalytic activity was further corroborated by assessing the transport kinetics of [3H]NE at both WT and hDAT mutants. [3H]NE-mediated transport by WT hDAT (data not shown) was saturable and of low affinity, with estimated K_m and V_max values of ~5 μM and ~3 pmol/10^6 cells/min, respectively, consistent with the affinity and lower translocation efficiency exhibited by hDAT for NE as compared with its preferred substrate, DA (34). hDAT mutants displayed a K_m for [3H]NE transport similar to that of WT hDAT (~4 μM, n = 2) with an observed slight reduction in apparent V_max (~2 pmol/10^6 cells/min). As such, DAT COOH-terminal truncation/substitution mutants appear to discriminate between dopamine and NE, inducing high affinity DA uptake while not affecting low affinity NE transport, unlike that seen in synaptosomes in which both DA and NE transport is of high affinity (28). Moreover, while we did not assess the kinetics of [3H]MPP^+, it would appear that there is no clear relationship between effects of DAT COOH-terminal truncation/substitution on substrate affinity (also see Table II) and the reported translocation efficiency of these compounds by hDAT.

Numerous studies have indicated that the cloned WT hDAT displays an expressed K_m for its endogenous substrate (K_m ~2 μM), at least 10-fold higher than that observed (K_m ~200 nM) in either rat or human native synaptosomes (28, 63–65). The observed discrepancies in estimated affinities for dopamine uptake are evident in either transient or stably expressing DAT cell lines of either neural or non-neural origin (19, 22, 28, 31, 66), indicating the limited contribution of local cellular microenvironments on transport. Although conceivably, the presence of endogenous dopamine in native preparations may contribute to the overestimation of affinity values, no such differences are observed for the cloned serotonin or γ-aminobutyric acid transporters, and in most published reports of the NE transporter (Refs. 23, 34, and 66–71; however, see Refs. 28 and 33). While the molecular mechanism is unknown, we document that the removal of the last 23 amino acids or substitution of the COOH tail of the hDAT confers or unmasks a high affinity transport component selective for the endogenous substrate dopamine, with an expressed K_m mimicking that seen in native synaptosomes. The possibility that the COOH-terminal tail of a small proportion of native neuronal DATs is similarly subject to some form of post-translational modification to effect the induction of high affinity dopamine uptake is intriguing. Although direct evidence for this contention is not available, it is of interest to note that glutamate transporters, upon exposure...
Numerous dopaminergic transport compounds were assessed for their ability to inhibit \[^{3}H\]dopamine uptake in COS-7 cells expressing either wild type or carboxyl terminal truncated/substituted hDAT mutants. Estimates for substrate and inhibitor K\(_i\) values for \[^{3}H\]dopamine uptake in COS-7 cells expressing either wild type or mutant transporters. K\(_i\) values reported are the means of three to six determinations, each performed in duplicate, with S.E. of less than 15%. K\(_i\) values are listed in their rank order of potency for \[^{3}H\]dopamine uptake for wild type hDAT. Since there were no statistically significant differences between the estimated K\(_i\) values for these compounds at either hDAT-D1 or hDAT-D5, both values were combined and listed as a single entry under hDAT-D1/D5.

### Table II

Estimated substrate and inhibitor K\(_i\) values for \[^{3}H\]dopamine uptake in COS-7 cells expressing either wild type or carboxyl terminal truncated/substituted hDAT mutants.

|Compound| \[^{3}H\]DA uptake: K\(_i\) (nM)|
|---|---|
|Amfonelic acid| 3| 1| 2| 1|
|Lu19-005| 6| 2| 2| 1|
|(+)-Diloctensine| 8| 8| 9| 4|
|Mazindol| 18| 14| 8| 9|
|CFT| 21| 9| 11| 5|
|Methylphenidate| 26| 18| 13| 18|
|Nomifensine| 33| 17| 23| 30|
|GBR-12909| 65| 61| 57| 48|
|d-Amphetamine| 100| 320| 360| 250|
|Buproprion| 520| 130| 410| 170|
|Dopamine| 1700| 170| 220| 150|
|Noradrenaline| 6800| 7100| 8100| 9000|
|MPP\(^{+}\)| 9300| 40,000| 20,000| 18,000|
|Serotonin| 43,000| 110,000| 36,000| 59,000|

Along with investigating the effects of COOH tail truncation/substitution on DA uptake, mutants were also assessed for their ability to exhibit high affinity ligand binding characteristics. As previously reported (19, 31) \[^{3}H\]CFT bound to COS-7 cells expressing WT DATs in a saturable and concentration-dependent manner to a single class of binding site with an estimated K\(_D\) of 22 ± 5 nM and B\(_{max}\) of 130 ± 10 fmoI/10\(^5\) cells (n = 4). Estimated K\(_D\) values for this compound at all hDAT mutants were reduced approximately 4–5-fold (80–110 ± 14 nM) with an observed concomitant doubling in the estimated number of binding sites (B\(_{max}\) to ~250 ± 41 fmoI/10\(^5\) cells (n = 3; data not shown). The accurate estimation of both the K\(_D\) and apparent B\(_{max}\) values for \[^{3}H\]CFT binding to all hDAT mutants was difficult to attain, however, due to limited ability to reach and use saturating concentrations of \[^{3}H\]CFT (up to 200 nM used). The density and affinity of binding sites labeled by \[^{3}H\]CFT were therefore evaluated in saturation experiments using cold CFT/\[^{3}H\]CFT. Nonlinear and Scatchard transformation of \[^{3}H\]CFT binding data to COS-7 cells expressing the WT hDAT (Fig. 4) revealed curvilinear functions indicative of two binding components and consistent with previous reports (29, 74, 75). Thus, estimated K\(_D\) and B\(_{max}\) values for \[^{3}H\]CFT binding to high and low affinity components were 55 ± 8 and 3500 ± 500 nM and 0.15 ± 0.006 and 1.24 ± 0.06 pmol/10\(^5\) cells, respectively. In contrast, computer-assisted nonlinear analysis and Scatchard transformation of \[^{3}H\]CFT binding to all hDAT mutants was found to be uniphasic and could not be resolved into multiple binding components (p > 0.05). As with estimates obtained by direct saturation experiments, the K\(_D\) of \[^{3}H\]CFT binding to hDAT-tr2 was significantly increased to 1.1 ± 0.5 μM with a concomitant 50% increase in the estimated B\(_{max}\) to 1.91 ± 0.08 pmol/10\(^5\) cells (n = 6). Virtually identical results were obtained for all other hDAT mutants (data not shown). While absolute K\(_D\) and B\(_{max}\) values for \[^{3}H\]CFT binding to either WT or mutant DATs are not identical between these two methods, the data are consistent with the suggestion that the relative ability of COOH tail-truncated hDATs to recognize \[^{3}H\]CFT with high affinity is severely compromised.

The ability of hDAT mutants to recognize and bind substrates and inhibitors with high affinity and with an appropriate pharmacological profile was also assessed. As illustrated in Fig. 5A, and as described previously (19), \[^{3}H\]CFT binding to WT hDAT transfected COS-7 cells is inhibited by dopamine in a biphasic manner with expressed high (−150 nM) and low (−6000 nM) affinity components. The proportion of sites recognized by dopamine as existing in either of these states is comparable (−50%). In marked contrast to the effects on dopamine uptake, the ability of dopamine to inhibit \[^{3}H\]CFT binding to all four hDAT mutants was markedly reduced. As depicted in Fig 5A, dopamine/\[^{3}H\]CFT competition curves were uniphasic, with an observed estimated K\(_i\) for dopamine (−10 μM) corresponding to the low affinity site states exhibited by WT hDAT. It appears that hDAT COOH tail truncation/substitution has abolished the existence of the dopamine high affinity \[^{3}H\]CFT binding component and reaffirms the contention that the observed modifications in ligand binding affinity are most likely due to the removal of COOH tail hDAT-specific sequences distal to Pro\(^{399}\). Other compounds that recognize both high and low affinity conformations of \[^{3}H\]CFT binding to
DAT, such as amphetamine and Lu 19-005, exhibited $K_i$ values for these sites/states similar to that of WT hDAT. Although there was a tendency for the proportion of high affinity sites recognized by these compounds to be reduced for all hDAT mutants, these did not reach statistical significance. Table III lists the estimated $K_i$ values and approximate proportions of both high and low affinity components for these compounds for $[3H]$CFT binding to WT and all hDAT mutants. In order to address the specificity of this result, GBR-12909 (Fig. 5 B) and several other hDAT inhibitors were also examined for their ability to inhibit $[3H]$CFT binding. Estimated $K_i$ values for these and all other compounds tested are listed in Table III. As is evident from the data, most of the compounds exhibited $K_i$ values for $[3H]$CFT binding to hDAT mutants that were at least ~10-fold greater than the $K_i$ exhibited by WT hDAT, with compounds such as mazindol and methylphenidate being particularly affected with estimated $K_i$ values 50-100-fold greater than that of WT DAT.

Taken together, the data obtained with hDAT COOH-terminal tail truncation/substitution mutants clearly suggest that the site or conformation of DAT required for the appropriate expression of substrate and inhibitor affinities at its ligand binding domain, at least as indexed by $[3H]$CFT binding, is functionally distinct from those mediating the recognition of these compounds for the DAT translocation process. A two-state recognition model has recently been proposed for the glutamate transporter (72) and is consistent with data depicting the dissociation of the dopamine uptake process from that of inhibitor recognition domains (32-34, 76). While the molecular mechanisms are still unknown, the data suggest that the ligand binding domain of hDAT may be regulated independently from the recognition/uptake process and may account for some anomalous observations suggesting the lack of a one to one correspondence between $[3H]$CFT binding and dopamine uptake (36).

In order to account for the dissociation of the dopamine translocation process from the ligand binding domain we assessed whether COOH tail truncation/substitution induces a rigid or "locked-in" (51, 52) conformational state of DAT. We reasoned that by coexpressing WT hDAT with hDAT-tr1, hDAT-tr2, hDAT-D1, and hDAT-D5, the wide disparity between WT and mutant DATs were uniphasic and best fit to a single site. Data shown are representative of four to six independent determinations each conducted in duplicate. Estimated $K_i$ values for these and other compounds are listed in Table III.
Table III

| Compound          | CFT          | Methylphenidate | Lu 19-005 | Mazindol | (+)-Dicyclominesine | GBR-12909 | Nonlensine | d-amphetamine | Dopamine | Cocaine | Buproprion | MPP + |
|-------------------|--------------|-----------------|-----------|----------|--------------------|-----------|------------|---------------|-----------|----------|------------|-------|
| Kinetic constants |              |                 |           |          |                    |           |            |               |           |          |            |       |
| Michaelis-Menten  | \(K_{m}\)    |                | \(V_{max}\) |            |                    |           |            |               |           |          |            |       |
|                   | \(\text{nm}\) |                 | \(\text{pmol} / \text{min}\) |            |                    |           |            |               |           |          |            |       |
| CFT               | 3500 (66%)   | 650 (84%)       | 750 (100%)| 40 (65%) | 20 (85%)          | 80 (90%) | 100 (90%) | 25 (95%)     | 150 (41%)| 180 (100%)| 3100 (60%)| 11000 (60%)|
| Methylphenidate   | 3 (45%)      | 500 (100%)      | 40 (65%) | 150 (60%)| 40 (85%)          | 120 (100)| 110 (90%) | 125 (90%)    | 150 (100)| 970 (100%)| 2600 (90%)| 100,000 (100%)|

Table IV

| Transfection condition | Ki (\text{nm}) | \(V_{max}\) (\text{pmol} / \text{min} / \text{cell}) |
|------------------------|----------------|-----------------|
| hDAT                   | 2400 ± 250     | 5.8 ± 1.2       |
| hDAT-tr1               | 180 ± 82       | 0.4 ± 0.06      |
| hDAT-tr2               | 12,150 ± 3955  | 4.8 ± 1.2       |
| hDAT-D1                | 250 ± 92       | 0.3 ± 0.07      |
| hDAT+D1                | 20,700 ± 9280  | 4.5 ± 0.6       |
| hDAT+D1-tr1            | 339 ± 103      | 0.4 ± 0.2       |
| hDAT+D1-tr2            | 20,800 ± 7400  | 5.4 ± 1.3       |
| hDAT+D1+D1             | 3100 ± 189     | 5.7 ± 1.0       |
| hDAT+D1+D1-tr1         | 3100 ± 329     | 4.9 ± 1.0       |
| hDAT+D1+D1-tr2         | 2325 ± 320     | 4.7 ± 0.6       |

hDAT-D1 was inhibited in a concentration-dependent and uniphasic manner by GBR-12909 with an estimated Ki value of 40 ± 9 nm, reflective of cells expressing WT hDAT protein alone (Ki, 32 ± 6 nm). No hint of the expected hDAT mutant Ki value for this compound (Ki = 324 ± 30 nm), being at least 10–20-fold lower than WT hDAT, was seen, even when [3H]CFT concentrations were increased to -50 nm.

To assess whether or not the lack of hDAT mutant activity was due to either an hDAT induced silencing of mutant transporter expression or some undefined recombination event upon co-transfection of both plasmid DNAs, COS-7 cells co-transfected with WT and hDAT-D1 were immunoblotted with polyclonal antibodies raised to epitopes within the amino-terminal of hDAT (57) and to COOH-terminal tail sequences of the human dopamine D1 receptor (58). As depicted in the inset to Fig. 6A, blots labeled with antibodies specific to the hDAT amino terminus indicated the presence of plasma membrane transporter expression in COS-7 cells transfected with hDAT, hDAT-D1, or both. The observed relative reduction of hDAT immunoreactivity in cells expressing hDAT alone is due to lower protein expression levels resulting from transfection of only 4 \mu g of DNA compared with 40 \mu g for hDAT-D1. Although not rigorously quantified, it appears that cells transfected with WT hDAT under these conditions exhibit a significantly greater loss in detectable membrane protein, as detected by immunoblotting, than can be accounted for by losses in cell surface transport activity or total number of membrane DAT ligand binding sites (see above). Little immunoreactivity was observed in cytosolic fractions (data not shown). Blots probed with D1 antibodies, however, only revealed immunoreactivity in COS-7 cell membranes expressing hDAT-D1 alone or hDAT and D1-D1. Mock-transfected cell membranes did not exhibit any detectable levels of immunoreactivity to either hDAT or dopamine D1 receptor antibodies. As such, despite the inability to observe mutant hDAT activity in cells co-expressing WT hDAT, both WT hDAT and mutant transport proteins appear to be processed and expressed in membranes of these cells.

One concept that may account for the observed lack of functional phenotypic co-expression of WT and mutant DAT activity, and in line with the observations described above, is that hDATs possibly oligomerize or form intermolecular associations to maintain expression of hDAT transporter activity. The formation of hetero- or homooligomeric complexes has been
Functional Role of DAT Carboxyl-terminal Tail

**Fig. 6.** Dopamine transport kinetics and [3H]CFT binding characteristics of COS-7 co-expressing wild-type and mutant DATs. COS-7 cells were transfected with 4 μg of WT hDAT, 40 μg of hDAT-D1, or both and assayed for [3H]dopamine uptake (A) with the indicated concentration of substrate as described under “Experimental Procedures.” Under these transfection conditions, uptake activities exhibited by cells expressing either WT or hDAT-D1 alone were similar. Data are representative of eight independent determinations, each conducted in duplicate. Identical results were obtained in COS-7 cells co-expressing WT and other DAT mutants with estimated V_max and K_m values listed in Table IV. Insert, representative dot blots depicting the immunodetection of WT hDAT, hDAT-D1, or both, by enhanced chemiluminescence. COS-7 cell membranes expressing WT hDAT, hDAT-D1, or both were blotted and probed with anti-hDAT (57) or anti-D1 (58) polyclonal antibodies as described under “Experimental Procedures.” The observed reduction of hDAT immunoreactivity in cells expressing hDAT alone is due to lower protein expression levels resulting from transfection of only 4 μg of DNA compared with 40 μg for hDAT-D1. Mock-transfected cell membranes did not exhibit any detectable levels of immunoreactivity to either hDAT or dopamine D1 receptor antibodies.

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demonstrated for members of distantly related transport families (77–79) regulating transporter function and stability. More pertinent to the present discussion, however, are radiation inactivation (80, 81) studies estimating the functional molecular mass of the DAT ligand binding domain to be considerably larger than that predicted by primary amino acid structure. While not entirely conclusive, the data support the notion that native DATs exist in at least a dimeric state, particularly for the maintenance of appropriate [3H]CFT binding characteristics (38). We suggest, therefore, that sequences within the COOH-terminal tail may possibly regulate the formation of hDAT multimeric subunits (82) and the loss of high affinity [3H]CFT binding interactions and expression of high affinity dopamine uptake characteristics by COOH tail-truncated hDATs is directly attributable to the inability of these mutants to form homo-, di-, or multimeric complexes. Although the stoichiometry is yet undefined, cells co-expressing both proteins may allow for the formation of heterodimeric hDAT-mutant subunit complexes by virtue of the presence of unmodified carboxyl-terminal tails on WT hDATs, resulting in the functional expression of what appears as only WT hDAT uptake and ligand binding characteristics. The necessity to reduce hDAT protein expression levels to a significantly greater extent than that observed for hDAT mutants to effect equivalence in the functional expression of DA uptake (see Fig. 6A, inset) circumstantially supports the view that WT DATs may exist in oligomeric states. A similar argument can be made with regard to the observed increase in the apparent B_max for [3H]CFT binding to hDAT mutants relative to WT protein. Moreover, the inability of β-mercaptoethanol (100 mM) to alter either WT or hDAT mutant dopamine uptake kinetics in cells (data not shown) further suggests that mechanisms governing DAT oligomerization are not associated with intermolecular covalent disulfide bond formation between extracellular hDAT domains. It would be of interest to determine whether these events are specific for DAT or possibly constitute a more generalized phenomenon, to include other sodium-dependent neurotransmitter transporters as well.

In summary, we provide evidence for a novel and previously unappreciated functional role of the COOH-terminal tail of hDAT. Thus, truncation/substitution of the carboxyl tail not only confers high affinity dopamine uptake mimicking that seen in native synaptosomes, but in addition abolishes appropriate and pharmacologically relevant [3H]CFT binding interactions, providing evidence for the complete functional dissociation of ligand binding recognition domain from the dopamine translocation process. Moreover, we tentatively suggest, based on data obtained with co-expressed WT and hDAT mutant transporters, that possibly one functional role of sequence-specific motifs of the hDAT carboxyl-terminal tail is to regulate or maintain the formation of hDAT oligomeric complexes. Future work will be directed at identifying those sequence-specific motifs and structural domains that may mediate this process and further our understanding of its role in the maintenance and regulation of presynaptic resting potentials and membrane excitability (83), hypo- or hyperdopaminergic disease states, and cocaine’s abuse liability (75, 84).

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