

A cognate dopamine transporter-like activity endogenously expressed in a COS-7 kidney-derived cell line¹

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Abstract The activity of the dopamine transporter is an important mechanism for the maintenance of normal dopaminergic homeostasis by rapidly removing dopamine from the synaptic cleft. In kidney-derived COS-7, COS-1 and HEK-293 but not in other mammalian cell lines (CHO, Y1, Ltk⁻), we have characterized a putative functional dopamine transporter displaying a high affinity ($K_m \sim 250$ nM) and a low capacity (~ 0.1 pmol/10⁵ cells/min) for [³H]dopamine uptake. Uptake displayed a pharmacological profile clearly indicative of the neuronal dopamine transporter. Estimated K_i values of numerous substrates and inhibitors for the COS-dopamine transporter and the cloned human neuronal transporter (human dopamine transporter) correlate well with the exception of a few notable compounds, including the endogenous neurotransmitter dopamine, the dopamine transporter inhibitor GBR 12,909 and the dopaminergic agonist apomorphine. As with native neuronal and cloned dopamine transporters, the uptake velocity was sodium-sensitive and reduced by phorbol ester pre-treatment. Two mRNA species of 3.8 and 4.0 kb in COS-7 cells were revealed by Northern blot analysis similar in size to that seen in native neuronal tissue. A reverse-transcribed PCR analysis confirmed the existence of a processed dopamine transporter. However, no immunoreactive proteins of expected dopamine transporter molecular size or [³H]WIN 35,428 binding activity were detected. A partial cDNA of ~ 1.3 kb, isolated from a COS-1 cDNA library and encoding transmembrane domains 1–6, displayed a deduced amino acid sequence homology of $\sim 96\%$ to the human dopamine transporter. Taken together, the data suggest the existence of a non-neuronal endogenous high affinity dopamine uptake system sharing strong functional and molecular homology to that of the cloned neuronal dopamine transporter.

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1. Introduction

The neuronal dopamine transporter (DAT), strategically

localized to presynaptic areas in distal dendritic and axonal processes of nigrostriatal neurons [1,2], regulates synaptic dopamine (DA) concentrations by the sodium-dependent re-uptake of DA, thereby terminating the dopaminergic input to pre-/post-synaptic DA receptors. Blockade of the neuronal DA transporter by cocaine, methylphenidate and other psychostimulants increases the extracellular DA that is available to act upon multiple pre- and post-synaptic receptors, eliciting psychomotor behavioral events. In DAT knockout mice, a behavioral phenotype is characterized by spontaneous hyperlocomotion and absence of any locomotor stimulating effect by psychostimulant drugs [3] due to the persistent duration of extracellular synaptic DA [4]. In addition, cocaine and amphetamine are ineffective on the DA release in these mice indicating that DAT is a primary target for these drugs, although some recent work has challenged the notion that DAT is an obligatory target for the abuse liability of these compounds [5,6].

The neuronal DA transporter has been cloned and belongs to the family of 12 transmembrane (TM) domain Na⁺/Cl⁻-dependent transporters (see [7–9]). While direct evidence for the existence of multiple DATs has not been reported, heterogeneity of the DA transporter is suggested by several observations (reviewed in [9,10]). It is not yet clear whether the observed pharmacological functional heterogeneity is a result of differential post-translational modifications of a single DA transporter [11] or due to the existence of multiple molecular species or multiconformational states of the transporter [12,13].

A dopaminergic uptake/transport mechanism is also expressed in the periphery. The presence of the DA transporter has been demonstrated in the pancreas [14] and in isolated parietal cells [15] by immunohistochemistry, while a transport system for L-dopa and DA is documented in kidney [16–18]. Although the characteristics of the kidney DA transport site are not concordant with the recognized pharmacology of the neuronal DAT [16,17], a dopaminergic system within the kidney is strongly supported by the presence of both D1-like and D2-like DA receptors whose functionality appears to be imperative for normal fluid homeostasis (see [19,20]). Peripheral DA, produced in proximal tubules from circulating L-dopa, can result in a renal vasodilatation and natriuresis via stimulation of DA D1-like receptors and blockade of a peripheral DA transport mechanism may, in part, account for cocaine-induced lethality that can be reduced by specific D1-like receptor antagonists [21,22].

To begin to assess the molecular nature of peripheral DA transport mechanisms, we report here the existence of a slow acting, high affinity DA transporter characterized in cell lines

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Abbreviations: DAT, dopamine transporter; hDAT, human dopamine transporter; NET, norepinephrine transporter; PMA, phorbol 12-myristate 13-acetate; GBR 12,909, 1-[2-bis(4-fluorophenyl)methoxyethyl]-4-(3-phenylpropyl)piperazine; WIN 35,428 (CFT), 2β-carbomethoxy-3β-(4-fluorophenyl)tropane; SCH-23390, R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; SKF-38393, (±)-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; NPA, propylnorapomorphine; 6,7-ADTN, (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene; TM, transmembrane

originally derived from the kidney (COS-7, COS-1 and HEK-293) that can be differentiated from the cloned neuronal DAT on the basis of its expressed pharmacological profile and mRNA processing.

2. Materials and methods

2.1. [^3H]DA uptake

Measurement of DA uptake was performed on intact COS-7, COS-1 or HEK-293 cells ($\sim 2 \times 10^5$ cells/well) plated in 24 well plates and grown in Dulbecco's modified Eagles serum containing 10% fetal bovine serum. COS-7, COS-1, HEK-293 and Ltk⁻ cells were all obtained from ATTC (Rockville, MD, USA). Briefly, confluent cells were washed with 0.5 ml buffer (5 mM Tris, 7.5 mM HEPES, 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). [^3H]DA uptake was initiated by the addition of the indicated concentrations of dopaminergic agents (10^{-11} – 10^{-4} M) and 10 nM (final concentration) of [^3H]DA (41.4–53.2 Ci/mmol, NEN/Dupont) and incubated in duplicate for 90 min at room temperature. Assays were terminated by rinsing twice with 0.5 ml buffer and solubilization of the cells with 1% SDS (0.5 ml/well) for 15 min at room temperature. Incorporated radioactivity was measured by liquid scintillation spectrometry in a Beckman LS 6000SC scintillation counter. Non-specific [^3H]DA uptake was defined in the presence of 10 μM mazindol and was typically less than 10% of the total. Estimated K_m and V_{\max} values for [^3H]DA uptake and K_i values were analyzed using the non-linear least squares curve fitting program KALEIDAGRAPH (Abelbeck Software).

2.2. Screening of a COS-1 λ ZAP cDNA library

Since the pharmacological profile of the endogenous DAT from COS-7 cells appeared to match the profile from COS-1 cells as did a Southern genomic DNA blot, a COS-1 λ ZAP cDNA library from Stratagene was used to clone this endogenous transporter. Briefly, duplicate nylon filters (NEN/Dupont) containing a total of $\sim 5 \times 10^5$ clones were screened under the conditions described [9] with an $\alpha^{32}\text{P}$ -labelled nick-translated full-length *EcoRI* 2 kb hDAT fragment (1×10^6 cpm/ml), washed twice in $2 \times \text{SSC}$, 1% SDS for 15 min 60°C and once in $1 \times \text{SSC}$, 1% SDS for 5 min at 60°C . Upon plaque purification of hybridizing clones, in vivo excision and rescue of the pBluescript SK⁻ plasmids containing the inserts, Southern blot analysis and sequencing, three weakly hybridizing clones were found to encode the creatine transporter while the fourth more intensely hybridizing clone, an *EcoRI* 1.3 kb fragment, contained a sequence homologous to the cloned DAT. Both strands of this clone were sequenced using the Sanger dideoxy chain termination method with 7-deaza-dGTP and Sequenase (USB) with either specific internal primers (Biotechnology Service Centre HSC, Toronto, Ont., Canada) or T7/T3 primers.

2.3. Northern blot analysis

Total RNA from COS-7 cells and Y1 (2.5×10^7 cells) was isolated using TRISOLV (Biotex) according to the manufacturer's directions. Approximately 30 μg of total RNA was run on a denaturing glyoxal/DMSO gel, transferred to a nylon membrane (Zeta-probe, Bio-Rad) and hybridized overnight at 42°C with 2.5×10^6 cpm of ^{32}P -labelled full-length hDAT as described previously [9]. The blot was washed once at room temperature for 15 min in $2 \times \text{SSC}$, 1% SDS, once at 65°C for 15 min in $2 \times \text{SSC}$, 1% SDS, once at 65°C for 15 min in $1 \times \text{SSC}$, 1% SDS and once at 65°C for 15 min in $0.5 \times \text{SSC}$, 1% SDS.

2.4. Reverse-transcribed (RT)-PCR analysis from COS-1 RNA

Since the clone from the library was prematurely truncated after TM6, 3'-RACE was performed using $\sim 1 \mu\text{g}$ total RNA to determine if the clone was a library artefact. Samples were treated with DNase for 15 min at room temperature prior to first strand cDNA synthesis with 25 pmol of the adapter primer containing an oligo dT sequence and 200 U Superscript reverse transcriptase (Gibco BRL). The single-stranded cDNA was then subjected to 30 cycles of PCR amplification (denature: 94°C , 1 min; anneal: 60°C , 1.5 min; extension: 72°C , 1.5 min) with 2 U *Taq* DNA polymerase (Gibco BRL) and 0.5 μg of a 5' internal primer (5'-GATTCACTGCAACAACCTCTG-3') and a 3' primer created from the sequence 3' to the putative stop after TM6 (5'-CTCCCTCACTGTCACTTCTGT-3'), transferred to a ny-

lon membrane and probed with a $\gamma^{32}\text{P}$ end-labelled oligonucleotide created from the sequence around TM 4 (5'-CAGAGGCTGAAGTA-GAGCAGCACGATG-3'). The blot was washed once at room temperature for 15 min in $2 \times \text{SSC}$, 1% SDS and once at 42°C for 15 min in $1 \times \text{SSC}$, 1% SDS. Two hybridizing bands (~ 670 bp and 540 bp) revealed on the autoradiograph were subcloned into pBluescript SK⁻ and sequenced. No bands were evident in the DNase controls.

2.5. Construction and expression of hDAT half mutants

TM 1–6 and TM 7–12 half mutants of hDAT were constructed by PCR and subcloned into the expression vector pCD-PS. For the TM 1–6 hDAT mutant, PCR primers corresponding to the region 5' to the start codon (5' primer: 5'-CAGCCCCAACTGCTCGGATGCC-CATCCTGGTG-3') and a PCR primer constructed from the loop before TM7 and incorporating a stop codon (3' primer: 5'-GTGCAATTCTTACCTGTAGCAGTTGTTGGT-3') were used. For the TM 7–12 mutant, the 5' primer before TM7 was constructed with an initiation methionine with a predicted Kozak sequence (Kozak, 1986) while the 3' primer was constructed after the stop codon (5' primer: 5'-GGTACCGCCGCCACCATGGACGCGATTGTCACC-ACC-3', 3' primer: 5'-TCTGTCCACCAGCTCA-3'). Approximately 200 ng of hDAT cesium-purified DNA was subjected to 30 cycles of PCR amplification (denature: 94°C , 1 min; anneal: 60°C , 1.5 min; extension: 72°C , 1.5 min) with 2 U *Taq* DNA polymerase (Gibco BRL) and 0.5 μg of the appropriate primers. Each half mutant was transfected in COS-7 cells by electroporation [9] or in Ltk⁻ by DEAE-dextran [23] and assayed for [^3H]DA after 10 min and 90 min to determine the functionality of the half mutant clones.

All other methods, including immunoblot analyses [24] and [^3H]CFT binding [9], were performed essentially as described.

3. Results and discussion

An uptake system for DA in the periphery, as suggested by some studies [14–16], would serve as a potential mechanism for regulating local DA concentrations, preventing accumulation within the general circulation. Using a kidney-derived cell line (COS-7) commonly used for transient expression functional studies of G protein-coupled receptors and the cloned neuronal DA transporter, we have characterized the pharmacological and molecular properties of a putative endogenous DA transporter with characteristics cognate to native neuronal DATs. As illustrated in Fig. 1A, the time course for [^3H]DA uptake in hDAT-transfected versus mock or untransfected cells was disparate with the endogenous COS-DAT differing with respect to initial levels of [^3H]DA uptake. Comparison of the time course for [^3H]DA uptake indicated that the cloned hDAT when expressed in COS-7 cells and the endogenous COS-DAT differed by more than 5-fold with hDAT being linear for up to 15 min, whereas the COS-DAT uptake was linear for up to 100 min. In contrast, other cell lines (CHO, Y1, Ltk⁻) did not show any detectable levels of endogenous [^3H]DA uptake, while the total [^3H]DA uptake for Sf9 cells was only $\sim 18\%$ of that observed for COS-7 cells (data not shown).

As depicted in Fig. 1B, [^3H]DA uptake measured in mock or untransfected COS-7 cells exhibited a high affinity with an expressed K_i of ~ 250 nM. In addition, saturation analysis indicated that COS-DAT displayed two affinity states for DA transport as determined by Eadie-Hofstee transformation. One site represented a high affinity uptake component with an estimated K_m of 130 ± 15 nM and a low translocation velocity as indexed by an estimated V_{\max} of 0.12 ± 0.01 pmol/ 10^5 cells/min. The other site consisted of a low affinity, high capacity component with an estimated K_m of 8.3 ± 0.9 μM and a V_{\max} of 0.64 ± 0.2 pmol/ 10^5 cells/min (Fig. 1B). Significantly, the observed high affinity K_m for [^3H]DA uptake is similar to

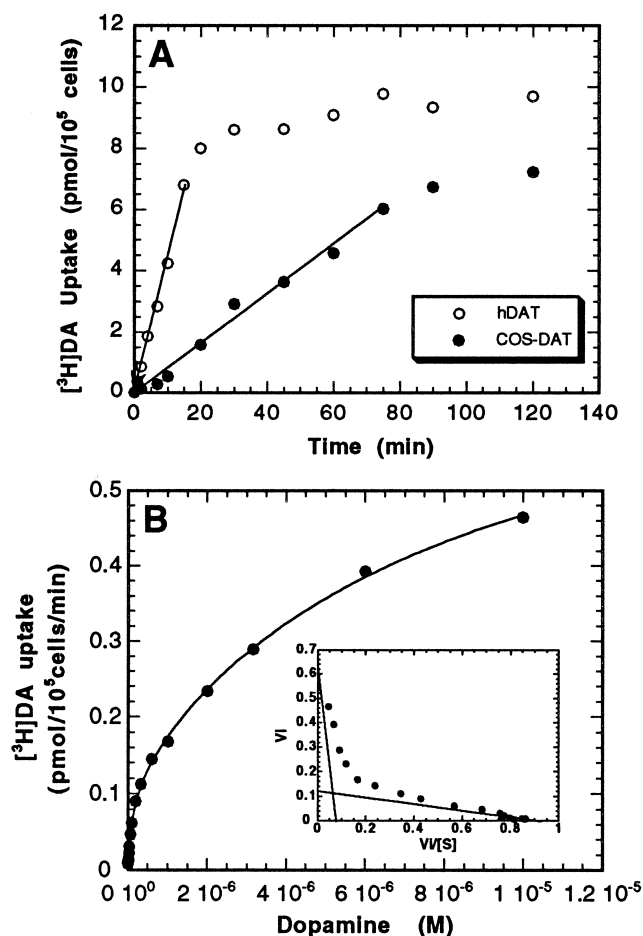


Fig. 1. Characterization of $[^3\text{H}]\text{DA}$ uptake by the endogenous COS-DAT. (A) Comparison of the time course of $[^3\text{H}]\text{DA}$ uptake for COS-DAT versus hDAT. Data are plotted as the amount of $[^3\text{H}]\text{DA}$ uptake measured at the time points indicated. Non-specific binding was defined in the presence of $10\ \mu\text{M}$ mazindol. (B) Representative saturation isotherm for $[^3\text{H}]\text{DA}$ uptake. COS-7 cells ($\sim 2.5 \times 10^5$) were incubated with $10\ \text{nM}$ $[^3\text{H}]\text{DA}$ and increasing concentrations of unlabelled DA for 90 min at room temperature as described in Section 2. Non-specific binding was defined in the presence of $10\ \mu\text{M}$ mazindol. Inset: Eadie-Hofstee transformation of saturation data. Data are representative of at least two independent experiments, conducted in duplicate and which varied by less than 15%.

the K_m value obtained from native synaptosomal preparations (50–280 nM) rather than that obtained for the cloned DAT ($\sim 1\text{--}5\ \mu\text{M}$) either expressed transiently or stably in a number of neuronal and non-neuronal cell lines (see [9,10]). The cloned human DAT, however, when expressed in Sf9 cells displayed an apparent affinity ($K_m \sim 260 \pm 60\ \text{nM}$) that corresponded to the K_m from striatal synaptosomes [10] and that reported here. Similarly, carboxyl-terminal tail truncations/substitutions of hDAT expressed in COS-7 cells also exhibited an estimated K_m of $\sim 300\ \text{nM}$ [24]. The presence of two distinct affinity components for the DA uptake, while not observed with full-length hDAT expressed in mammalian or Sf9 cells [9,10], was, however, clearly evident in carboxyl-terminal tail-truncated hDATs expressed in COS-7 cells with estimated K_m values of 210 nM and $18.9\ \mu\text{M}$ [24]. This suggests perhaps that DAT sequence-specific motifs particularly within the carboxyl-terminus in conjunction with the local cellular micro-

environment may account for the observed differences in K_m values. Interestingly, high affinity $[^3\text{H}]\text{DA}$ uptake was also observed in two other kidney-derived cell lines, COS-1 and HEK 293, with a total uptake of 67% and 43%, respectively, compared to COS-7 cells. Each displayed an estimated K_m value for DA at the high affinity component similar to that for COS-7 cells ($130 \pm 15\ \text{nM}$ and $280 \pm 30\ \text{nM}$, respectively). In these cell lines, the DA uptake was sodium-sensitive as indicated by a $\sim 80\%$ reduction in total uptake by replacement of NaCl with LiCl (data not shown).

As illustrated in Fig. 2A, $[^3\text{H}]\text{DA}$ uptake was inhibited by dopaminergic compounds in a concentration-dependent, ster-

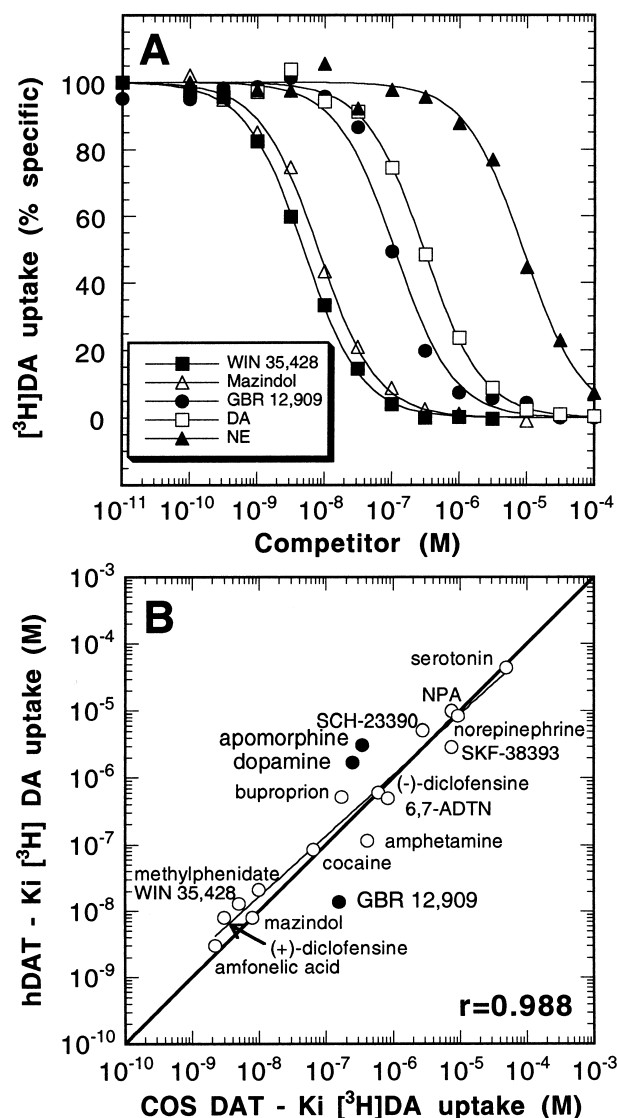


Fig. 2. Pharmacological profile of the endogenous COS-DAT. (A) Representative curves of the inhibition of $[^3\text{H}]\text{DA}$ uptake in COS-7 cells. Cells ($\sim 2 \times 10^5$ /well) were incubated with $10\ \text{nM}$ $[^3\text{H}]\text{DA}$ and the indicated concentration of competing agents (10^{-11} – $10^{-4}\ \text{M}$) and assayed for $[^3\text{H}]\text{DA}$ uptake activity as described in Section 2. Estimated K_i values are listed in Table 1. Data are representative of at least two or three independent experiments, each conducted in duplicate and which varied by less than 15%. (B) Correlation plot of estimated K_i values for the $[^3\text{H}]\text{DA}$ uptake for COS-DAT compared to hDAT. Estimated K_i values for inhibition of $[^3\text{H}]\text{DA}$ uptake by a series of DAT uptake inhibitors and substrates for hDAT were taken from ([9,24]). A line of identity is shown.

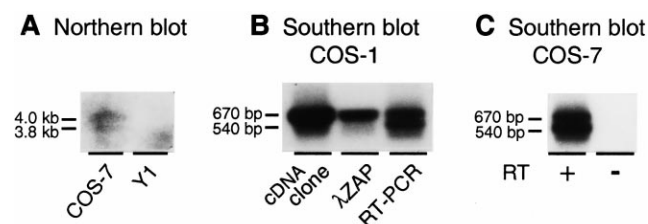


Fig. 3. Characterization of endogenous COS-DAT-like transporter. (A) Northern blot analysis of COS-7 and Y1 RNA. Total RNA ($\sim 30 \mu\text{g}$) isolated from COS-7 and Y1 cells using TRISOLV was denatured, electrophoresed on a 1% agarose DMSO/glyoxal gel, transferred to a nylon membrane and probed with a ^{32}P -labelled full-length hDAT fragment. The sizes of the hybridizing bands are indicated to the left. (B) Southern blot of RT-PCR-amplified products from COS-1. Samples of COS-1 total RNA were subjected to reverse transcriptase (RT) using oligo (dT) and then to PCR with two additional COS-1 DAT-specific oligonucleotide primers (see Section 2). Amplified products from the RT-PCR analysis and from PCR analysis off the cDNA clone and COS-1 λZAP II library were Southern-blotted and probed with a COS-1 DAT-specific ^{32}P -labelled oligonucleotide primer internal to the PCR products. Sizes of hybridizing bands (bp) are indicated. (C) Southern blot of RT-PCR-amplified products from COS-7. COS-7 total RNA was subjected to reverse transcriptase using oligo (dT) followed by PCR amplification with the same two COS-1 DAT-specific oligonucleotide primers. Amplified products were Southern-blotted and probed with a COS-1 DAT-specific ^{32}P -labelled oligonucleotide primer internal to the PCR products. The same two hybridizing bands (~ 670 bp and ~ 540 bp) are evident in COS-7 cells. DNase control with no reverse transcriptase (RT) is located to the right. Sizes of hybridizing bands (bp) are shown.

eselective and uniphasic manner (as determined by Hill coefficients close to unity). Estimated K_i values for several dopaminergic substrates and inhibitor compounds (listed in Table 1) for [^3H]DA uptake in COS-7 cells correlate extremely well with K_i values obtained from the cloned hDAT [9,24] or with native neuronal DATs. As depicted in Fig. 2B, only a few compounds, the exceptions noted, differentiated the cognate COS-DAT from the expressed cloned hDAT, namely DA and apomorphine which exhibited a ~ 10 -fold higher affinity for COS-DAT. In contrast, GBR 12,909 displayed a between 5- and 10-fold lower affinity for COS-DAT than expected. The observed differences in the pharmacological profiles along with the detected differences in the initial rates of DA uptake between cloned and cognate DATs in COS-7 cells were used to confirm the observed heterogeneity in pharmacology of the two transporters. Thus, when hDAT was transfected into COS-7 cells and assayed for [^3H]DA uptake after 10 min, the standard time period for transfected DAT uptake assays, the K_i value for apomorphine was approximately 1920 ± 280 nM. This is in contrast to the K_i value of 354 ± 87 nM for the endogenous DAT or the somewhat intermediate K_i of 715 ± 98 nM for hDAT-transfected COS-7 cells assayed after a 90 min incubation period (data not shown). Thus, the endogenous COS-DAT under the appropriate experimental conditions can functionally contribute or modulate the pharmacological characteristics of the cloned hDAT. DA uptake was not, however, inhibited by L-dopa (up to $100 \mu\text{M}$) or by the NE uptake blocker desipramine ($10 \mu\text{M}$), nor could the presence of a high affinity [^3H]NE uptake system in COS-7 cells be detected. The inability to observe [^3H]NE uptake (10 nM) may be related to the rather low density of high affinity COS-DATs and the reduced translocation velocity exhibited

by DAT for non-preferred substrates [25]. In line with this observation is the fact that no specific [^3H]WIN 35,428 (CFT) binding was observed in control or mock-transfected whole cell assays or prepared membranes [9,24] despite the ability of this compound to inhibit the DA uptake with a high affinity. It appears therefore that the sites mediating the appropriate expression of substrate or inhibitor affinities at the ligand binding domain of COS-DAT-like molecules, at least as indexed by [^3H]WIN 35,428 (CFT), are distinct from those conferring the recognition of these compounds for the DA translocation processes. Similar observations have been made with the cloned human DAT (e.g. [24] and references therein).

Similar to other Na^+/Cl^- -dependent transporters [26–28], the cognate COS-DAT appeared to be regulated by second messenger activation of protein kinase C. Thus, after pretreatment with $1 \mu\text{M}$ PMA, a protein kinase C activator, for 30 min, a significant drop ($\sim 80\%$) in V_{max} for DA occurred with no large change in apparent K_m ($\sim 700 \pm 150$ nM). No such a reduction in V_{max} was procured after pretreatment with $10 \mu\text{M}$ forskolin (104% of control total [^3H]DA uptake) suggesting that protein kinase A does not modulate the activity of this transporter, similar to the lack of effect of 8-Br-cAMP on DA uptake into striatal synaptosomes [29,30] or on DAT phosphorylation [31]. In both native striatal synaptosomes and heterologous expression systems, the DA uptake (V_{max}) is decreased when treated with PMA with no apparent change in K_m [24,30,32–34]. The exact nature of the decrease in the apparent DA translocation velocity after protein kinase C activation is unknown but may be due to the rapid sequestration of the DAT protein from the cell surface as demonstrated by immunofluorescent confocal microscopy [10].

The presence of a functional COS-7 DA transporter with characteristics quite similar to native neuronal or cloned DATs predicted the presence of a DAT mRNA transcript in these cells. As illustrated in Fig. 3A, Northern blot analysis of

Table 1
 K_i values (nM) for inhibition of [^3H]DA by COS-DAT

Compound	COS-DAT (nM)	COS-DAT/hDAT
Amfonelic acid	2.2	0.73
(+)-Diclofensine	3.0	0.38
WIN 35,428	4.9	0.38
Lu 19,005	5.7	0.46
Mazindol	8.0	1.0
Methylphenidate	10.3	0.48
Cocaine	65	0.76
GBR 12,909	159	11.3
Bupropion	171	0.33
DA	250	0.15
Apomorphine	354	0.18
Amphetamine	416	3.59
(-)-Diclofensine	605	1.00
6,7-ADTN	843	1.80
SCH-23390	2 800	0.54
NPA	7 500	0.75
SKF-38393	7 600	2.62
Norepinephrine	9 500	1.11
Serotonin	> 50 000	> 1

K_i values for inhibition of [^3H]DA uptake are listed in order of potency. Data represent the means of at least 2–4 independent experiments, each conducted in duplicate and which varied by less than 15%. Ratios were determined using the K_i values from [9,34] for COS-7 cells transiently expressing hDAT.

total RNA extracted from COS-7 cells and probed with full-length ^{32}P -labelled hDAT indicated the presence of two lightly hybridizing bands of ~ 3.8 and 4.0 kb. This is in agreement with the estimated transcript size of the rat (~ 3.7 kb, [35]) and the human neuronal DA transporter (~ 4.2 – 4.5 kb, [36,37]). As expected, no discernible hybridizing bands could be detected in Y1 cells, as these cells do not display any high affinity uptake of [^3H]DA (data not shown). The presence of multiple COS-DAT mRNA species may be indicative of splice variants or different transcription start or polyadenylation sites [38]. To ascertain whether DAT-like protein are present and maturely processed in these cells, membranes of untransfected COS-7 and COS-7 cells expressing hDAT were subjected to Western blotting with polyclonal antibodies to hDAT [1]. However, no specific immunoreactive bands were present in untransfected COS-7 cells. Moreover, no immunoreactive labelling of untransfected COS-7 cells could be visualized by confocal microscopy [10].

In an attempt to determine the molecular nature and deduced amino acid composition of the processed COS-DAT, a commercially prepared COS-1 cDNA library was screened under medium stringency conditions with full-length hDAT. A COS-1 cell line library was chosen since COS-1 displayed endogenous DAT-like functional activity and Southern blotting of *EcoRI*, *SacI*, *PstI*, *XhoI* and *HindIII* restriction-digested genomic COS-1 and COS-7 DNA with a ^{32}P -labelled hDAT yielded identical hybridization patterns (data not shown). Four hybridizing clones were isolated, three of which produced a weak hybridization signal and upon sequence analysis were determined to encode the creatine transporter, cloned previously from kidney [39]. The fourth clone (*EcoRI* 1.3 kb fragment) which hybridized intensely with hDAT was found by sequence analysis to contain a deduced amino acid sequence¹ virtually identical (96%) to hDAT. However, quite surprisingly, while the clone contained a poly A sequence at the 3'-end preceded by ~ 500 bp 3'-UTR, the clone appeared to be prematurely truncated as indexed by the presence of a stop codon following TM6 at exon/intron boundary 7. No clones containing TM 7–12 were obtained from this library as revealed by re-screening the library with a fragment encoding TM8 to the carboxyl-terminus of hDAT. RT-PCR analysis was therefore performed on COS-1 and COS-7 RNA using an internal 5'-specific oligonucleotide primer within the encoding sequence and a 3' oligonucleotide primer constructed from the isolated 3' untranslated cDNA sequence. As illustrated in Fig. 3B and C, two amplified cDNA products of apparent sizes, ~ 670 bp and ~ 540 bp, were revealed. Upon sequence analysis, the 670 bp fragment yielded the expected sequence with a stop codon after exon 7 (encoding TM6) identical in position to the putative stop codon seen with the cDNA clone isolated from the library, while the 540 bp product revealed that exon 6 (encoding TM5) was missing. Taken together, these results indicate that the COS-1 cDNA DAT clone, at least at the 3'-end of the clone, is not simply a product of aberrant library construction and suggests that under appropriate conditions and in a cell-specific manner, preferential processing of 'truncated' forms of the DAT are evident in both COS-1 and COS-7 cells. Similar to other members of this transporter family [40–43], the DA transporter gene contains several exons separated by intronic sequences [37,44], suggesting the possibility of alternative splicing. The human serotonin transporter appears to be alternatively

spliced within the 5' non-encoding regulatory region [43] while alternative mRNA splicing of the norepinephrine transporter (NET) gives rise to proteins with variant carboxyl-termini that differ with respect to their functional expression [42,45]. Alternatively, the inability to acquire a full-length clone may be due to the low abundance of this transporter mRNA in these kidney-derived cell lines.

Since we were unable to find a full-length clone from the COS-1 library or obtain evidence for a corresponding truncated version of COS-DAT protein via immunodetection, we assessed whether truncated DA transporters can confer the uptake characteristics observed in these cells. Recent studies have suggested that truncated GLUT1 glucose transporters (TM 1–6 and TM 7–12) can re-aggregate to form functional proteins [46]. Two hDAT mutants were generated by PCR, one encoding sequence beginning with the initiation methionine and terminating within the third intracellular loop (Arg-344) at the identical position to that seen with the isolated COS-1 cDNA clone and the other hDAT 'half' mutant encoding a Kozak sequence with an initiation methionine sequence that has been shown to naturally occur in one reported clone of the human hDAT sequence [47] followed by the remaining carboxyl-terminal half (TM7-CT end) of the protein. [^3H]DA uptake was assessed in either COS-7 or Ltk⁻ cells following transient expression of either mutant alone or after co-expression to determine if the two halves of the transporter could re-assemble to form a functional transporter. The half mutants, either expressed individually or concurrently, could not demonstrate any specific [^3H]DA uptake above that which occurs endogenously in these cells when assayed at either 10 or 90 min, thereby indicating that these two half mutants were not functional (data not shown).

In summary, we report the characterization of an endogenous DA transporter in several kidney-derived cell lines (COS-7, COS-1 and HEK-293) and a partial cDNA clone from COS-1 cells that shares functional, pharmacological and molecular characteristics of the cloned neuronal DAT. The presence of a functional DAT-like activity in these cells, although a low capacity, confirms the reported presence of DAT proteins within the periphery [14,15] and suggests that peripherally re-uptake of DA may serve as an additional mechanism for regulating DA concentrations. The isolation of a prematurely truncated DAT-like molecule indicates that the DA transporter may be subjected to alternative processing depending on the cell type. However, functional analyses of truncated hDAT mutants suggest that these truncated forms do not mediate DA uptake. Possibly, these forms may require additional ions, co-transporters or transport-adaptor proteins to mediate uptake or, alternatively, may function only as releasers. In any event, we have confirmed the existence of a peripheral DAT, as suggested by immunohistochemical studies. The further localization of DAT within the periphery may indicate a more widespread role for the DAT function.

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