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The *Caenorhabditis elegans* Gene *T23G5.5* Encodes an Antidepressant- and Cocaine-Sensitive Dopamine Transporter

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ABSTRACT

A small subset of neurons in the nematode *Caenorhabditis elegans* utilizes the catecholamine dopamine (DA) as a neurotransmitter to control or modulate movement and egg-laying. Disruption of DA-mediated behaviors represents a potentially powerful strategy to identify genes that are likely to participate in dopaminergic systems in man. In vertebrates, extracellular DA is inactivated by presynaptic DA transport proteins (DATs) that are also major targets of addictive agents, including amphetamines and cocaine. We used oligonucleotides derived from the *C. elegans* genomic locus *T23G5.5* to isolate and characterize T23G5.5 cDNAs. Our studies predict that mRNAs from this locus encode a 615-amino-acid polypeptide with twelve stretches of hydrophobicity suitable for transmembrane domains, similar to that found in vertebrate catecholamine transporters. The inferred translation product bears highest identity (43–47%) to catecholamine (DA, norepinephrine, epinephrine) transporters within the *GAT1/NET* gene family and possesses conserved residues implicated in amine substrate recognition. Consistent with these findings, HeLa cells transfected with the *C. elegans* cDNA exhibit saturable and high affinity DA transport (K_m = 1.2 μM) that is dependent on extracellular Na^+ and Cl^- and blocked by inhibitors of mammalian catecholamine transporters, including norepinephrine transporter- and DAT-selective antagonists, tricyclic antidepressants, and the nonselective amine transporter antagonists cocaine and D-amphetamine. These studies validate the *T23G5.5* locus as encoding a functional catecholamine transporter, providing important comparative sequence information for catecholamine transporter structure/function studies and a path to identify regulators of dopaminergic signaling via genetic or pharmacologic manipulation of *C. elegans* cDNA *in vivo*.

Chemical signaling by small molecule neurotransmitters, including glutamate, glycine, GABA, DA, NE, and 5HT, is terminated by transporter-mediated clearance (Rudnick and Clark, 1993). Pharmacologic or genetic disruption of transporter function elevates extracellular neurotransmitter levels, perturbs presynaptic transmitter homeostasis, and can trigger significant alterations in behavior (Giros *et al.*, 1996; Pelham, 1997). The psychoactive agents cocaine and the amphetamines compete with substrates at amine (DA, NE, 5HT) transporters, with much of their addictive potential attributed to DAT blockade (Kuhar *et al.*, 1991). In contrast, NE and 5HT transporter (NET and SERT, respectively) antagonists such as imipramine, desipramine, fluoxetine, and sertraline are important agents in the treatment of mood disorders, particularly depression (Barker and Blakely, 1995). The cloning of a rat GAT1 (Guastella *et al.*, 1990) and a human NET (Pacholczyk *et al.*, 1991) established the presence of a Na^+ and Cl^--dependent transporter gene family whose members include transporters for most small neurotransmitters except *l*-glutamate. Although significant ad-

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ABBREVIATIONS: GABA, γ-aminobutyric acid; DA, dopamine; NE, norepinephrine; 5HT, 5-hydroxytryptamine (serotonin); DAT, dopamine transporter; NET, norepinephrine transporter; SERT, serotonin transporter; GAT, γ-aminobutyric acid transporter; TMD, transmembrane domain; CeDAT, *Caenorhabditis elegans* dopamine transporter; PCR, polymerase chain reaction; RT, reverse transcriptase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase pair(s); ORF, open reading frame; Epi, epinephrine; bp, base pair(s).

vances have been made in recent years in identifying regions and residues involved in transporter-selective recognition of neurotransmitters and antagonists (Giros and Caron, 1993; Barker *et al.*, 1998), there remain significant challenges in understanding the structural basis for the transport process and how transporters are regulated by genetic and post-translational mechanisms. Furthermore, we have an imperfect understanding of the complex chain of biochemical and electrophysiologic responses after chronic transporter blockade (Hyman, 1996; Mongeau *et al.*, 1997), which dictates the development of novel strategies to decipher key addictive and therapeutic drug responses.

Biogenic amine transporter homologs isolated from invertebrates (Corey *et al.*, 1994; Demchyshyn *et al.*, 1994) offer important opportunities to exploit sequence divergence for structure/function studies (Barker and Blakely, 1997) and to implement genetic strategies to identify regulators or downstream targets of transporter genes and proteins. Studies with the *Drosophila melanogaster* SERT have revealed a prominent 5HT-gated ion channel activity (Galli *et al.*, 1997) and analyses of fly/human SERT chimeras have identified specific residues involved with 5HT and antagonist recognition (Adkins *et al.*, 1997; Barker *et al.*, 1998). Genetic disruption of *Drosophila* SERT has yet to be achieved; thus, it remains to be determined whether the complexity of the adult fly nervous system and its behavioral output will allow exploration of interacting genes via enhancer/suppressor screens. In contrast, genetic screens based on compromised 5HT and acetylcholine inactivation in *Caenorhabditis elegans* have revealed a number of important genes involved in neural differentiation and synaptic transmission (Desai and Horvitz, 1989; Miller *et al.*, 1996). The important role of DA as a modulatory neurotransmitter for movement and egg-laying behaviors (Huang *et al.*, 1982; Schafer and Kenyon, 1995) suggest additional opportunities to exploit *C. elegans* genetics based on pharmacologic or genetic (Zwaal *et al.*, 1993) manipulation of genes responsible for DA inactivation. As yet, however, a transporter or enzymatic pathway supporting DA clearance in *C. elegans* has not been identified.

Recently, the *C. elegans* genome sequencing project has revealed the presence of multiple *C. elegans* genes homologous to the *GAT1/NET* gene family of neurotransmitter transporters (Wilson *et al.*, 1994). The inferred translation of one of these genes, *T23G5.5* on chromosome 3, exhibits highest similarities to the biogenic amine subgroup of transporters in the gene family. Importantly, conceptual translation of the inferred amino acid sequence of *T23G5.5* reveals an Asp residue in TMD1 known to be conserved and functionally important for transport activity in DATs, NETs, and SERTs (Kitayama *et al.*, 1992; Barker and Blakely, 1995). To establish the validity of the conceptual translation of *T23G5.5* and to determine the functional specificity of the encoded transporter, we cloned cDNAs arising from this locus and expressed them in mammalian cells to define substrate selectivity and antagonist sensitivity. Our studies reveal that the *T23G5.5* locus encodes a cocaine- and antidepressant-sensitive catecholamine transporter, the substrate selectivity of which suggests that it represents the *C. elegans* DA transporter (CeDAT). We also identify a number of high affinity CeDAT antagonists that may be useful in future structure-function studies with mammalian DATs and NETs as well as for pharmacologic perturbation of nematode behavior that could be used in genetic screens.

Materials and Methods

cDNA library screening. An oligo(dT) primed cDNA library in lambdaZAP (Barstead and Waterson, 1989) was screened by conventional plaque hybridization (Sambrook *et al.*, 1989) using oligonucleotides matching DNA sequences reported in COSMID CET23G5 (Wilson *et al.*, 1994) associated with a hypothetical transporter gene (*T23G5.5*). Oligonucleotide RB 436 (sense, 5′-TAACCGCATTCTATGTGGATTTC-3′, exon 2) and RB437 (antisense, 5′-GTTGCACAATTGATGAATGATGTG-3′, exon 7) were synthesized by the Vanderbilt DNA core, precipitated, and end-labeled with [γ-^{32}P]ATP (Amersham, Arlington Heights, IL) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Probes were purified away from unincorporated nucleotides by gel filtration (NucTrap; Stratagene, La Jolla, CA) and hybridized to plaques on Magna nylon filters (Micron Separations, Westborough, MA) at 55° in 5× standard saline/phosphate/EDTA, 0.5 mg/ml heparin, 0.5% sodium dodecyl sulfate for 3 hr. Filters were washed twice at room temperature in 5× standard saline/phosphate/EDTA (750 mM NaCl, 57.5 mM NaH_2PO_4, 5 mM EDTA), 0.1% sodium dodecyl sulfate for 5 min, followed by a single 55° wash in the same solution, the filters were air-dried and exposed to X-ray film (Kodak XAR; Eastman Kodak, Rochester, NY). Positive plaques were cored from master plates, eluted in SM media (1× = 100 mM NaCl, 50 mM Tris, 8 mM $MgSO_4$, 0.01% gelatin, pH 7.5), and rescreened until single plaques were identified. Insert sizes were estimated using PCR on 0.4 μl of boiled phage stock using T3 and T7 sequencing primers (45° for 1 min, 72° for 3 min, 30 cycles). Inserts were excised as pBluescript SK^+ plasmids via *in vivo* plasmid rescue (Ex-Assist kit; Stratagene). Inserts were sequenced with vector and internal primers using fluorescent dye terminators on an ABI 310 automated DNA sequencer (ABI, Foster City, CA). Sequence contigs, alignments, and analyses utilized Lasergene for the Apple Macintosh (DNAstar, Madison, WI).

RT-PCR of *C. elegans* RNA. Isolated cDNAs lacked sequences complementary to the amino-terminus of known *GAT1/NET* family members as well as sequences matching inferred 5′ exons of the *T23G5.5* locus. Thus we amplified an initial 5′-end of the transporter's mRNA using RT-mediated PCR. Total *C. elegans* RNA was prepared with Trizol reagent (Sigma, St. Louis, MO) according to manufacturer's recommendations and analyzed on 6% formaldehyde-agarose gels (Sambrook *et al.*, 1989). RNA was further treated with RQ1 RNase free DNase (Promega, Madison, WI) before RT-PCR. RT-PCR was conducted with random priming of cDNA (Perkin-Elmer Cetus, Norwalk, CT) using oligonucleotides for PCR that match inferred exonic sequences. Our first sense primer (RB452: 5′-CAAATCTTCAGACGATCCCGACGAA-3′) was located toward the 5′ end of the hypothetical initiation codon of the T23G5.5 gene product. The antisense primer (RB453: 5′-CTAGGATAATGAAAGTGGAAGACAC-3′) was designed from sequence of our phage clone that, although not complete on its 5′ end, extended past a presumptive translation termination codon and established a significant extent of the transporter's ORF, including novel 3′ sequences (pCEGTl1; Fig. 1A). Using these primers, we amplified a single PCR product (pCeDAT1; Fig. 1A) (Qiaquick resin; Qiagen, Chatsworth, CA) for ligation into the vector pGEMTEasy (Promega). Multiple clones bearing the cDNA insert were isolated and sequenced as described above. Missense mutations caused by PCR in the 3′ end of the cDNA were corrected by transfer of a nonmutant *Nco*I fragment from a nonmutant PCR isolate. A single PCR-induced missense mutation in the 3′ end of the cDNA was corrected by oligonucleotide-mediated, site-directed mutagenesis (QuikChange System; Stratagene).

To obtain additional 5′ cDNA sequences, a set of nested RT-PCR reactions was conducted using an SL1 primer (GCAGGATCCGGTTTAATTACCCAAGCTTGAG), matching the 5′ *trans*-spliced leader

sequence found on the majority of *C. elegans* mRNAs (Blaxter and Liu, 1996), and two nested antisense primers (RB 515: 5′-AGTCCAGCTTTCCAGACCACTGTTC-3′ and RB516: 5′-CTGGCCG-AGGCACAACTCCATGTAG-3′), complementary to sequences in the 5′ end of pCeDAT1 (Fig. 1A). After sequence of this product to validate its *T23G5.5* and CeDAT origin, we reamplified this fragment with a sense oligonucleotide complementary to sequences immediately adjacent to the SL1 primer. We added an *Sph*I restriction site to the 5′ end of this primer (RB517: 5′-GCGCGCGCATGCTCCATATTCCAAATTAGTCGAAAAGCT-3′) to permit the use of an internal *Sph*I site within the 5′ RT-PCR product and pCeDAT1 to construct pCeDAT2 (Fig. 1A). The completed pCeDAT2 5′ end was sequenced to verify proper insertion and a lack of PCR-generated sequence errors.

Transient expression of CeDATs in HeLa cells. To determine whether isolated cDNAs encode functional transporters in transfected cells, we utilized the vaccinia-T7 expression system (Blakely *et al.*, 1991). pCeDAT DNA was prepared using Qiagen DNA isolation columns and mixed with Lipofectin (Life Technologies, Grand Island, NY) in a 1:3 (w/v) ratio at the time of transfection. HeLa cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies), 10% fetal bovine serum (Hyclone, Logan, UT), 100 μg/ml penicillin (Sigma), and 100 units/ml streptomycin (Sigma) at 37° in 5% CO_2. One day before transfections, 1×10^5 cells were plated in 24-well tissue culture plates. Medium was removed and cells washed with sterile, phosphate-buffered saline. Vaccinia-virus suspensions were added in OptiMEM (Life Technologies) medium (50 μl) to cells and allowed to infect for 30 min at 37° followed by application of pCeDAT liposomes diluted in OptiMEM/0.45% 2-mercaptoethanol (450 μl). Cells were assayed 6–12 hr after transfections for induction of transport relative to plasmid vector or nontransfected cells. Transport assays were conducted at 37° in 500 μl of final volume of Krebs-Ringer-HEPES buffer (120 mM NaCl, 10 mM HEPES, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.2 mM CaCl2, 10 mM D-glucose) containing 100 μM ascorbic acid (Sigma), 100 μM pargyline (Sigma) and 10 μM U-0251 (Upjohn Laboratories, Kalamazoo, MI). Radiolabeled substrates were initially examined at 20 nM and included [^{3}H]DA, [^{3}H]NE, [^{3}H]Epi, or [^{3}H]5HT (Amersham). Inhibition assays were initiated by addition of [^{3}H]DA (50 nM; Amersham) with or without varying concentrations of competitors. Amine transporter antagonists were preincubated with cells for 10 min before addition of substrate. Ion-dependence of [^{3}H]DA transport (50 nM) was inspected in assays substituting Li^+ and *N*-methyl-D-glucamine for Na^+ and gluconate for Cl^-. Assays were terminated by three ice-cold washes in Krebs-Ringer-HEPES before solubilization of cells in EcoScint and direct quantitation of accumulated radioactivity in a Microbeta scintillation counter (Wallac, Gaithersburg, MD). Data were analyzed using Kaleidagraph software (Synergy Software, Reading, PA), using a nonlinear, least-squares curve fitting algorithm to fit concentration response curves and determine IC_{50} values of competitors. K_i values were estimated from IC_{50} values using the Cheng-Prusoff correction for substrate concentration.

Results and Discussion

Using oligonucleotides derived from proposed exons 2 (RB 436) and 7 (RB437) of *T23G5.5* (Wilson *et al.*, 1994), we screened a lambdaZAP *C. elegans* cDNA library by conventional plaque hybridization techniques. No positive isolates (600,000 plaques screened) were obtained with the exon 2 probe, whereas multiple positive plaques were identified with the exon 7 probe (300,000 plaques screened). This suggests that the cDNA library available to us, which was oligo(dT) primed with an average insert size of 1 kb, was poorly

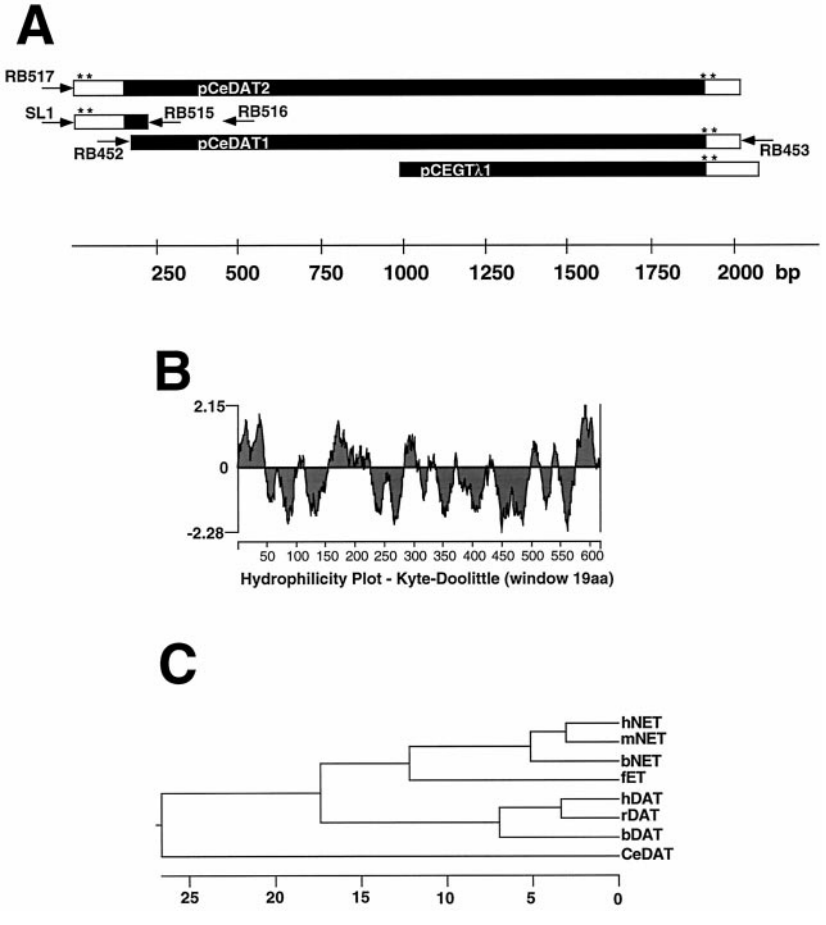

Fig. 1. Isolation and analysis of CeDAT cDNAs. A, cDNAs isolated or amplified in this study. RB452, 453, 517, and SL1 represent PCR primers used on *C. elegans* cDNA to identify partial CeDAT cDNAs as described in Materials and Methods. PCeDAT2 was constructed by splicing sequences amplified with RB517 and RB516 into pCeDAT1 at an internal *Sph*I site. *, position of in-frame stop codons. *Shaded areas*, ORF whose sequence is provided Fig. 2. B, Kyte-Doolittle hydrophilicity plot of the protein predicted by the ORF of pCeDAT2. Hydrophobic sequences likely to form TMDs are evident as downward peaks. C, Predicted phylogenetic relationships of CeDAT protein with other catecholamine transporters in the *GAT1/NET* gene family. The length of each horizontal line is proportional to the relative distance between transporters, with units indicating the number of substitution events, a measure of phylogenetic distance (DNAstar).

represented in T23G5.5 cDNAs having complete 5′ ends. PCR of plaque purified phage identified with the exon 7 probe using T3 and T7 oligonucleotide primers yielded an insert of ~1.1 kb for the largest insert, again suggesting a partial cDNA insert relative to the full length of the estimated T23G5.5 coding sequence (~1800 bp). Plasmid excision (clone pCEGTl1) and direct DNA sequencing confirmed the identity of these sequences as *T23G5.5* with the 5′ end beginning in hypothetical exon 6 (1177 bp; Fig. 2, *bracketed*). The 3′ end of the isolated cDNA was found to match the genomic sequence of cosmid CET02C1. However, sequence from this cDNA and subsequent RT-PCR products (see below) established that the hypothetical exon splicing pattern originally predicted in GENBANK for the 3′ end of the *T23G5.5* transcript is incorrect. Rather, we found that the CeDAT transcript is spliced from cosmid CET23G5 sequences at bp 26895 to cosmid CET02C1 sequences at bp 465 using canonical gt/ag donor/acceptor sites rather than at base 301. This gives the inferred protein a longer carboxyl terminus (Fig. 2), following the amino acid sequence RGNTISE with a set of 32 additional amino acids, including three residues that are conserved in catecholamine transporters from nematode to man (R587, Y592, P596; Fig. 3). Such strict conservation of these three amino acids in an otherwise highly divergent carboxyl terminus suggests that they may serve to stabilize secondary structure of this domain required for recognition by accessory proteins or regulatory protein kinases (Blakely *et al.*, 1998). The new stop codon is followed in the cDNA (and genomic sequence) by 227 bp of 3′ noncoding sequence that includes a canonical polyadenylation signal (AATAAA), located 19 bp upstream of a nongenomic poly(A)$^+$ tail, which suggests that the polyadenylated 3′ end of the CeDAT transcript has been identified.

To clone additional 5′ sequences associated with *TG235.5* and to establish a complete ORF suitable for expression in transfected cells, we designed oligonucleotide primers for amplification of *C. elegans* RNA via RT-PCR. Initially, we designed a sense primer (RB452) located just upstream of the predicted initiator methionine in *TG235.5* and an antisense primer 3′ (RB453) located distal to the stop codon identified in the corrected 3′ end of the TG235.5 transcript (Fig. 1A). These oligonucleotides amplified a 1.9-kb cDNA from random primed *C. elegans* total RNA (pCeDAT1; Fig. 1A). This RT-PCR product was gel purified, subcloned, and multiple plasmid clones were sequenced. Two clones (pCEGRTPCR1 and pCEGRTPCR5) were found to be oriented for sense RNA expression by the plasmid T7 RNA polymerase promoter and were sequenced in their entirety. Both cDNAs were found to have 1–3 nonidentical point mutations that could be established as PCR artifacts using pCEGTl1, multiple PCR subclones, and the sequence of cosmids CET23G5 and CET02C1. Missense mutations in pCEGRTPCR1 were corrected using nonmutated segments of RTPCR subclones and by site-directed mutagenesis to yield the expression construct pCeDAT1.

Although pCeDAT1 induced significant DA transport activity in transfected cells, no in-frame stop codons lay between the 5′ PCR primer used to create pCeDAT1 and the predicted initiator methionine; thus, further efforts were needed to validate the most likely initiation site for translation and establish the 5′ end of CeDAT mRNAs. We turned to RT-PCR, this time using a sense primer complementary to the SL1 leader RNA that is *trans*-spliced to the 5′end of the majority of *C. elegans* mRNAs (Blaxter and Liu, 1996) and nested antisense primers downstream of the 5′ end of CeDAT1 (Fig. 1A). The fragment amplified using the SL1 strategy identified a new exon in the TG235.5 gene from bp 23763 to bp 23850 in cosmid CET23G5. Canonical gt/ag splice junctions splice this new first exon into exon 2 at bp 24227 (377-bp intron) just upstream of the original, mispredicted initiator methionine. The new exon adds an additional 19 amino acids to the transporter's amino terminus in frame with the sequence originating at the previous CeDAT1 starting methionine (Fig. 2). This sequence was transferred to pCeDAT1 as described in Material and Methods to create pCeDAT2. Although two in-frame stop codons precede the new start codon (GCC*ATG*C) of pCeDAT2 and the new start codon conforms reasonably well to the Kozak consensus sequence ((A/G)CCATG(G)) for translation initiation (Kozak, 1986), we cannot rule out that translation could begin under some circumstances at Met20 as this site also possesses a good consensus sequence (GGCATGC) and pCeDAT1 expresses in transfected cells. No evidence was found in our 5′ RT-PCR reactions for alternative splicing of mRNA encoding the transporter's amino terminus.

The composite sequence of the predicted CeDAT mRNA drawn from our RT-PCR and phage isolates and the inferred translation of CeDAT protein is provided in Fig. 2. We predict that CeDAT is a 615-amino-acid polypeptide. Kyte-Doolittle hydrophilicity analysis suggests twelve hydrophobic stretches suitable for formation of TMDs that are well aligned with similar profiles of transporters in the *GAT1/NET* gene family (Figs. 1B and 2). Two canonical sites for *N*-linked glycosylation are located in the large hydrophilic loop between TMDs 3 and 4, sites analogous to those known to be glycosylated in mammalian catecholamine transporters (Melikian *et al.*, 1996). Additional *N*-glycosylation sites are evident in the transporter's amino (N22) and carboxyl (N597) termini, although direct evidence suggests that analogous regions of mammalian transporter homologs are intracellular (Brüss *et al.*, 1995) and the carboxyl terminal motif is preceded by a proline residue (P596) that typically renders the Asn inaccessible by the glycosylation machinery (Kornfeld and Kornfeld, 1995). The amino and carboxyl termini possess a number of Ser and Thr residues that may be targets for regulatory phosphorylation with two PKC sites (Ser45, Ser582) and one casein kinase II site (Thr580) among these. A cAMP-dependent protein kinase site (Ser255) also lies in a putative intracellular loop between TMDs 4 and 5 within a span of residues (WKGXXTSGKVVW) found in all catecholamine transporters (Figs. 2 and 3). Similarly, a casein kinase II site between TMDs 6 and 7 lies in a highly conserved stretch of sequence (A(Y/F)SSYN(D/K)F. Studies are underway to evaluate the role of these domains and residues in acute CeDAT regulation.

Comparisons with other *GAT1/NET* family members demonstrates highest similarity of CeDAT to mammalian catecholamine transporters. CeDAT exhibits ~47% amino acid identity with human, mouse, and bovine NETs, ~43% identity with human, bovine, and rat DATs, ~37% identity with human, rat and, mouse SERTs, and less than 35% identity with other gene family members. Sequence divergence suggests the carrier may have arisen from a common ancestral transporter before DATs, NETs, and ETs formed genetically

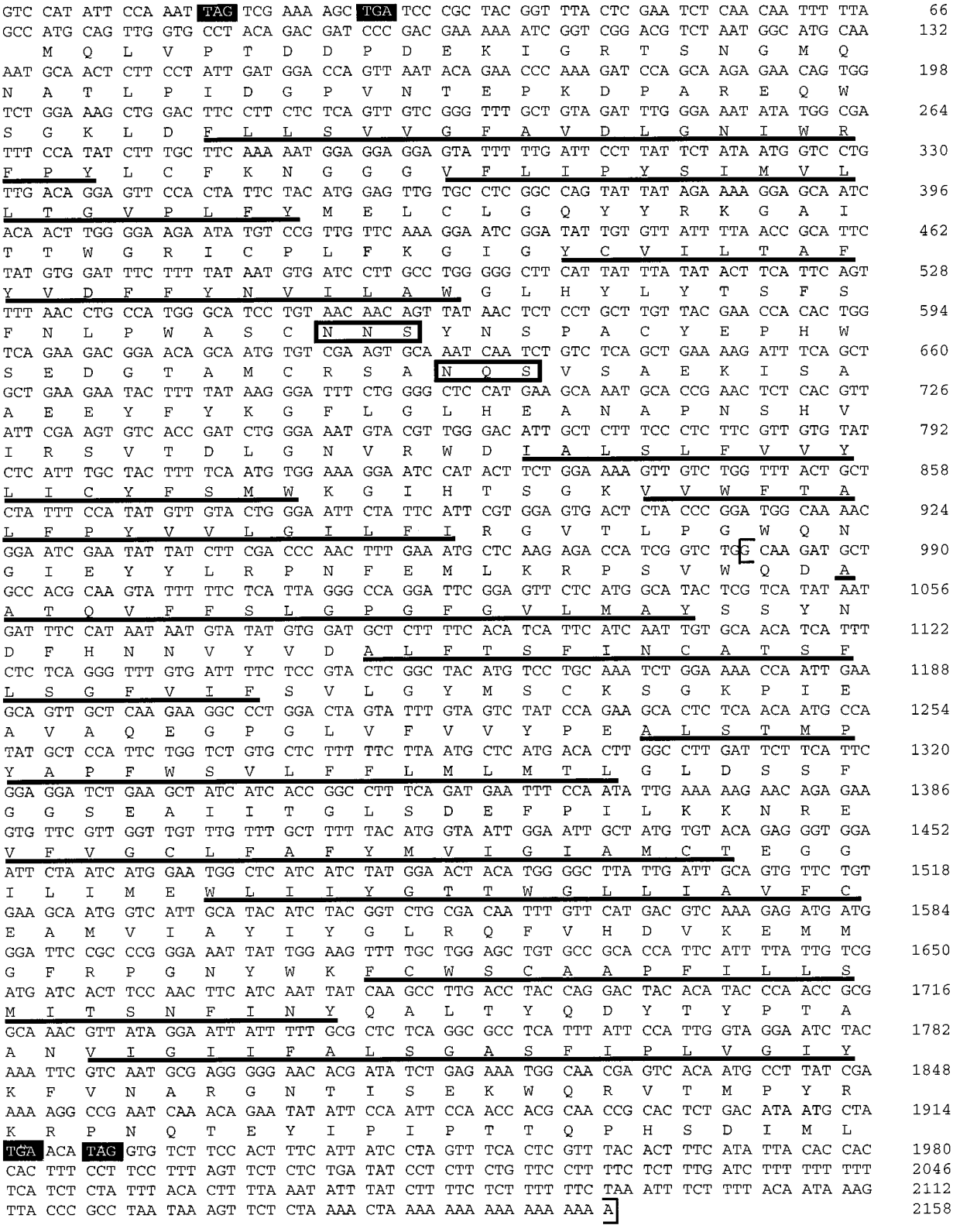

Fig. 2. CeDAT cDNA and protein (GenBank accession no. AF079899): composite cDNA sequence compiled from pCeDAT2 and pCETl1. *Reversed sequence*, in-frame stop codons relative to the open-reading frame translated; *lines*, estimation of the positions of transmembrane domains from hydrophilicity analysis and comparisons with other family members; *boxed sequences*, canonical *N*-glycosylation sites in the predicted extracellular domain separating TMD3 and 4. *Sequence in brackets*, that encoded by pCETl1. Numbers reflect bp length of cDNA beginning at the SL1 primer to the poly(A)$^+$ tail.

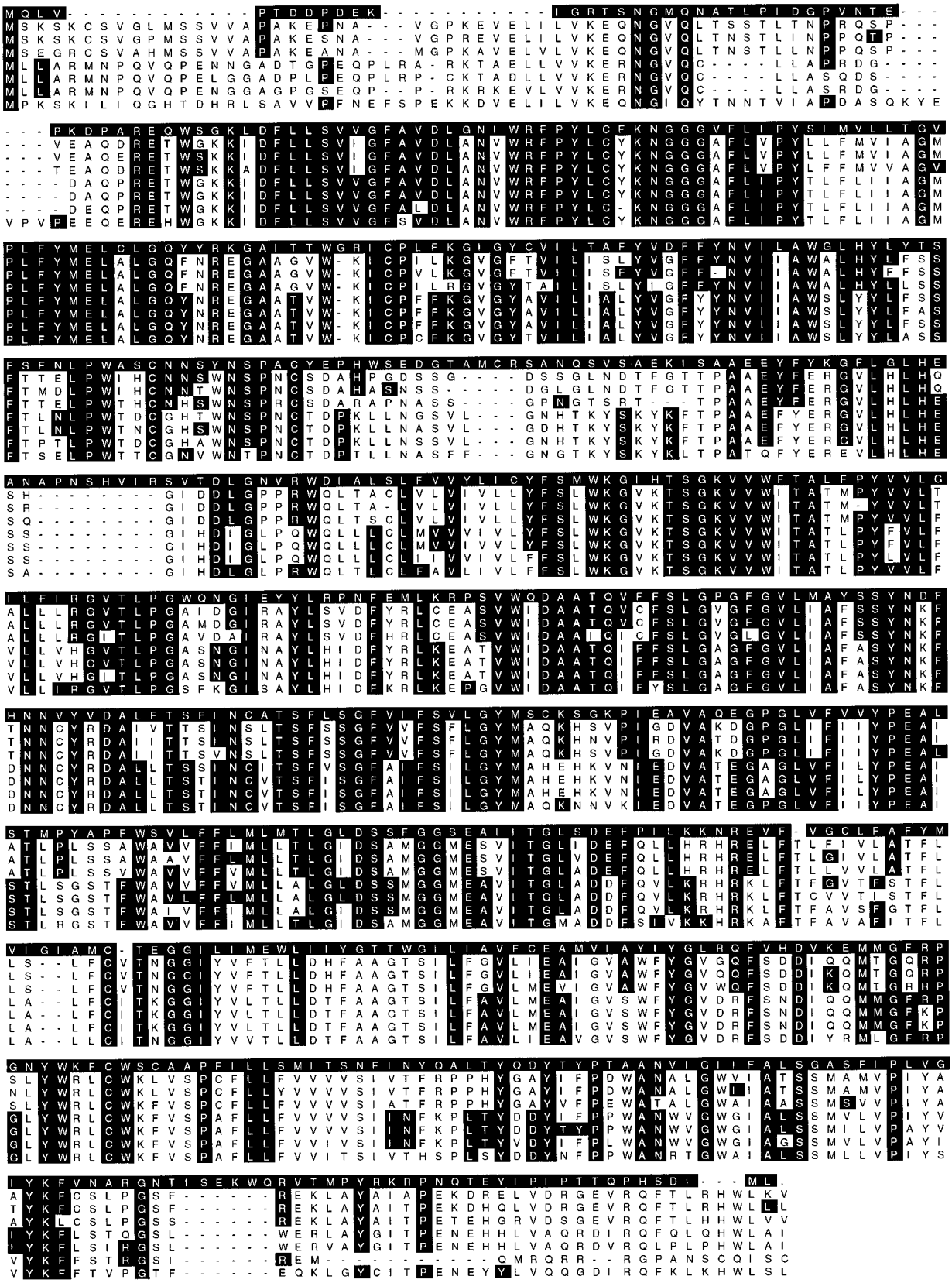

Fig. 3. Sequence relationships among catecholamine transporters. Depicted is an alignment of all known full-length species variants of catecholamine transporters. *Shading*, identity of amino acid sequence with CeDAT. *hDAT*, human DAT; *rDAT*, rat DAT; *bDAT*, bovine DAT; *hNET*, human NET; *mNET*, murine NET; *bNET*, bovine NET.

distinct species (Fig. 1C). An Asp residue that is conserved in TMD1 of the DA, NE, and 5HT transporters from fly to man (Kitayama *et al.*, 1992; Barker and Blakely, 1995) but absent from GABA, glycine, taurine, proline, creatine and taurine transporters, is also in the analogous position (D60) in CeDAT (Fig. 3), and originally suggested to us a functional specification of CeDAT for transport of amine substrates. The sequence divergence and conservation that is evident comparing CeDAT with its most closely related mammalian homologs should be useful in pinpointing critical residues for substrate and antagonist selectivity through future chimera and site-directed mutagenesis studies.

To establish the substrate selectivity of CeDAT, we used the vaccinia-T7 transient expression system previously employed in the characterization of mammalian *GAT1/NET* family members (Pacholczyk *et al.*, 1991). Initially, we tested radiolabeled DA, NE, Epi, and 5HT each at 50 nM because CeDAT bears the greatest sequence identity with mammalian amine transporters in the *GAT1/NET* gene family. Using pCeDAT1, which presumably initiates translation at Met 20, we found transfected HeLa cells to transport DA to a greater extent (~2 fold) than NE, with little or no transport of Epi and 5HT (data not shown). Using pCeDAT2, we repeated these studies and found overall higher expression levels and a maintained preference for DA over NE (Fig. 4A), whereas

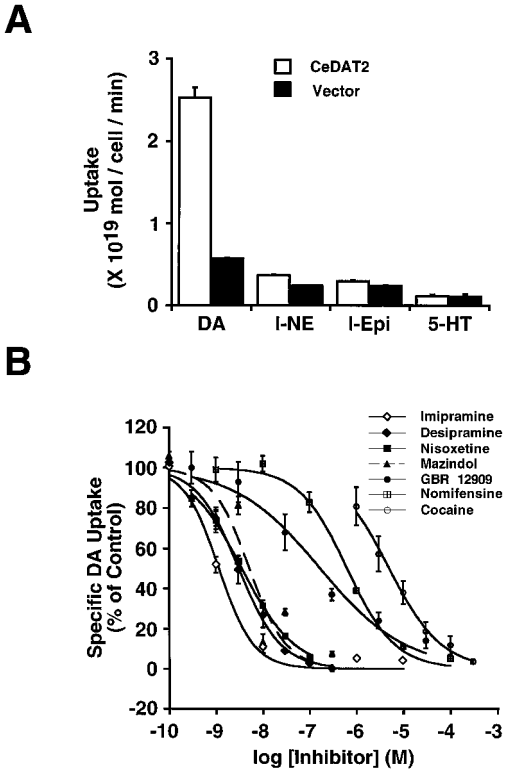

Fig. 4. Functional characterization of CeDAT in transiently transfected HeLa cells. A, Substrate selectivity assessed using either pCeDAT2 transfected cells or vector (pGEMTEasy) transfected cells. Values are mean of three transfections presented ± standard deviation. All ^{3}H-labeled substrates were assayed at a single (20 nM) concentration as described in Materials and Methods. Ordinate legend reflects multiplication of our data values by 10^{19} to achieve integer values on the axis. B, Inhibition of [^{3}H]DA transport induced by pCeDAT2 using unlabeled amine transporter antagonists. [^{3}H]DA was used at 50 nM. *Curves*, derived from three separate inhibition experiments; *points*, mean ± standard error with nonlinear curve fits obtained using Kaleidagraph software as described in Materials and Methods.

Epi and 5HT remained transported only at very low levels. The increased DA transport activity of pCeDAT2 versus pCEDAT1 may reflect a more efficient use of the translation initiation site present in pCEDAT2. The ability of CeDAT to transport 5HT to a small degree (not evident on the scale used for Fig. 4A) is not unanticipated as mammalian NETs do so as well (Blakely RD, unpublished observations). Indeed, a transporter homolog distinct from CeDAT and more closely related to mammalian SERTs has appeared in the nematode genome project databases and initial genetic studies of its locus are supportive of a role in 5HT clearance (Horvitz HR, personal communication). Transport of DA by CeDAT was completely abolished when Na^+ was substituted by Li^+ or *N*-methyl-D-glucamine (data not shown). Similarly, specific DA transport induced by CeDAT transfection was eliminated in Cl^- free media (data not shown). Thus, CeDAT exhibits a reliance on both external Na^+ and Cl^- ions for transport of DA as seen with mammalian DA transporters.

Given our initial results with likely amine substrates, we chose to use [^{3}H]DA and unlabeled competitors to explore relative potencies of substrate analogs and antagonists in competing for catecholamine transport. We found that DA (K_i = 146 nM) is almost a 10-fold more potent inhibitor of transport than *l*-NE (K_i = 1180 nM), consistent with their relative K_m values. *l*-Epi was a weak inhibitor of [^{3}H]DA transport (K_i = 3.4 μM). Tyramine (K_i = 827 nM) and octopamine (K_i = 67 μM) were also much less potent than DA as competitors of transport activity. Octopamine is suspected to be a neurotransmitter in invertebrates including *C. elegans* (Kravitz, 1988), although its low potency against CeDAT indicates this carrier is unlikely to be involved in its clearance *in vivo*. D-Amphetamine competed for DA transport at micromolar concentrations (K_i = 3.3 μM) and was more potent than the *l*-isomer (K_i = 13 μM), typically a selectivity associated with the mammalian *l*-NE transporter. Finally, 5HT competed for DA transport only at high micromolar concentrations (K_i >100 μM). Next, we explored the sensitivity of pCeDAT1 induced [^{3}H]DA transport to conventional amine transport antagonists (Barker and Blakely, 1995). Nanomolar concentrations (Fig. 4B) of the mammalian NET-selective antagonists nisoxetine and desipramine (K_i = 3 nM) as well as the DAT/NET-selective antagonist mazindol (K_i = 8 nM) inhibited CeDAT activity. Low concentrations of the DAT-selective antagonist GBR12909 (K_i = 170 nM) also blocked CeDAT activity. However, although the potent human NET antagonist nomifensine blocked CeDAT activity (K_i = 630 nM), although this is nearly 2 orders of magnitude less potent than required to inhibit human NET (Pacholczyk *et al.*, 1991). The relatively human SERT-selective tricyclic imipramine was a very potent antagonist of CeDAT activity (K_i = 1 nM), which suggests that a component of the compound's behavioral activity in nematodes may be nonserotonergic (Weinshenker *et al.*, 1995) and mediated through the DA system. Transport could also be blocked by the nonselective amine transporter antagonist cocaine (K_i = 5 μM), a potency similar to that seen at heterologously expressed DATs (Kilty *et al.*, 1991). Overall, these findings suggest that CeDAT has a unique profile of inhibitor sensitivity, sharing pharmacologic properties with both mammalian NETs, DATs, and SERTs. Further studies should be able to extend these pharmacologic profiles and, with comparative sequence analyses,

provide important avenues to the identification of substrate and antagonist contact sites.

The true substrate for CeDAT may best be inferred from the relative maximal capacities to transport different substrates. Mammalian NETs exhibit higher V_{max} values for NE versus DA whereas the reverse is true for DATs. Similarly, the frog Epi transporter transports substrates with a maximal rank order velocity of Epi > NE ≫ DA (Apparsundaram *et al.*, 1997). However, all three carriers exhibit greatest inhibitory potency for DA, suggesting that K_i or K_m values can be less informative than V_{max} rank order as to the identity of native substrates. Thus, we performed saturation kinetic studies on pCeDAT1 transfected cells and tested the relative capacities for transport for NE and DA, the two substrates exhibiting greatest transport activity at low substrate concentrations. We found DA to exhibit a lower substrate K_m and a higher transport V_{max} than NE, suggesting that in *C. elegans*, DA is likely to be the preferred substrate (Fig. 5, A and B). There are eight DA synthesizing neurons in *C. elegans* hermaphrodites (Sulston *et al.*, 1975) and, although early reports suggested the presence of NE and Epi (Kisiel *et al.*, 1976), no studies to date have identified the enzymes required to make these catecholamines (dopamine B-hydroxylase and phenylethanolamine *N*-methyltransferase, respectively) in the worm. Finally, recent cosmid and T23G5.5 promoter fusions reveal selective reporter expression in DA neurons (Ishihara T, Katsura I, and Hope IA, personal communication). Together these data indicate the *T23G5.5* transcript encodes the transporter responsible for DA inactivation *in vivo*.

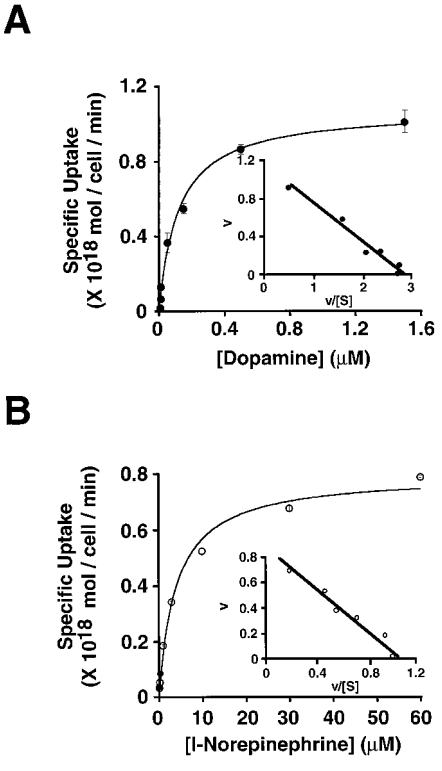

Fig. 5. Saturation kinetics of catecholamine transport induced by pCeDAT in transfected HeLa cells. Cells were transfected with pCeDAT1 and assayed in parallel with increasing concentrations of labeled [^{3}H]DA (A) or [^{3}H]NE (B) as described in Materials and Methods. Ordinate legends in A) and B) reflect multiplication of our data values by 10^{18} to achieve integer values on the axis. Specific uptake was defined by subtraction of activity obtained in vector transfected cells assayed in parallel. *Insets*, Eadie-Hofstee replots of saturation data (axis units, cell/min/liter × 10^{24}). K_m value for [^{3}H]DA was 1.2 μM and V_{max} was 1.08 pmol/10^6 cells/min. K_m value for [^{3}H]NE was 4.1 μM and V_{max} was 0.79 pmol/10^6 cells/min.

Mice that lack DATs exhibit markedly reduced clearance of released DA, the animals are hyperactive and they are insensitive to the locomotor-stimulant properties of cocaine and amphetamines (Giros *et al.*, 1996). Mice deficient in the 5HT transporter exhibit significantly diminished 5HT levels and have altered responses to addictive amphetamines (Pelham, 1997). Flys with mutations in the gene *inebriated* display altered motor coordination in response to anesthetics, although the substrate for this *GAT1/NET* homolog remains unknown (Soehnge *et al.*, 1996). Loss of one of the major glial glutamate transporters (GLT1) leads to seizures and neuropathology, perhaps through excitotoxic mechanisms (Tanaka *et al.*, 1997). These studies remind us of the important control that transporters exert over synaptic function. DA in *C. elegans* exerts powerful inhibitory control over movement and egg laying (Huang *et al.*, 1982; Schafer and Kenyon, 1995; Weinshenker *et al.*, 1995). Thus we anticipate that genetic or pharmacologic disruption of CeDAT *in vivo* will potentiate dopaminergic activity by preventing normal clearance and disrupt movement and reproduction. Specific CeDAT antagonists derived from the structures of imipramine, nisoxetine, or mazindol or other leads identified in high-throughput screens using heterologously expressed CeDAT may be very useful as antiparasitic agents. The biologic activity of such agents should also permit the development of novel screens to identify transporter regulatory genes. In addition, screens based on the phenotype of CeDAT blockade or genetic disruption may identify genes linked specifically to altered DA neurotransmission, among which may be homologs of genes triggered by excess DA availability in cocaine and amphetamine abuse in man.

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