0026-895X/01/5901-83–95$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 59:83–95, 2001

Vol. 59, No. 1
485/875639
Printed in U.S.A.

The Antidepressant-Sensitive Dopamine Transporter in *Drosophila melanogaster*: A Primordial Carrier for Catecholamines

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Received August 16, 2000; accepted October 10, 2000

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Extracellular concentrations of monoamine neurotransmitters are regulated by a family of high-affinity transporters that are the molecular targets for such psychoactive drugs as cocaine, amphetamines, and therapeutic antidepressants. In *Drosophila melanogaster*, cocaine-induced behaviors show striking similarities to those induced in vertebrate animal models. Although a cocaine-sensitive serotonin carrier exists in flies, there has been no pharmacological or molecular evidence to support the presence of distinct carrier subtypes for other bioactive monoamines. Here we report the cloning and characterization of a cocaine-sensitive fly dopamine transporter (dDAT). In situ hybridization demonstrates that dDAT mRNA expression is restricted to dopaminergic cells in the fly nervous system. The substrate selectivity of dDAT parallels that of the mammalian DATs in that dopamine and tyramine are the preferred substrates, whereas octopamine is transported less efficiently, and serotonin not at all. In contrast, dDAT inhibitors display a rank order of potency most closely resembling that of mammalian norepinephrine transporters. Cocaine has a moderately high affinity to the cloned dDAT (IC_{50} = 2.6 μM). Voltage-clamp analysis of dDAT expressed in *Xenopus laevis* oocytes indicates that dDAT-mediated uptake is electrogenic; however, dDAT seems to lack the constitutive leak conductance that is characteristic of the mammalian catecholamine transporters. The combination of a DAT-like substrate selectivity and norepinephrine transporter-like inhibitor pharmacology within a single carrier, and results from phylogenetic analyses, suggest that dDAT represents an ancestral catecholamine transporter gene. The identification of a cocaine-sensitive target linked to dopaminergic neurotransmission in *D. melanogaster* will serve as a basis for further dissection of the genetic components of psychostimulant-mediated behavior.

The mammalian monoamine transporters for dopamine (DA), norepinephrine (NE) and serotonin (5HT) are the sites of action for two major classes of psychoactive drugs: psychomotor stimulants and antidepressant medications. Psychostimulants like cocaine and amphetamines are potent inhibitors of all three monoamine transporters; however, most of their reinforcing properties and abuse potential have been attributed to the blockade of the dopamine transporter (DAT) (Ritz et al., 1987). Antidepressant medications, including the selective serotonin reuptake inhibitors and the tricyclic antidepressants, predominantly block the serotonin and norepinephrine transporters (SERT and NET) (Barker and Blakely, 1995), but have decidedly lower potencies for the mammalian DATs.

Functional disruption of the monoamine transporter genes in mice result in profound behavioral (Giros et al., 1996) and neurochemical (Bengel et al., 1998; Xu et al., 2000) changes and demonstrate the crucial role of the transporters in maintaining low extracellular amine concentrations in vivo. Progress in our understanding of changes induced by cocaine and antidepressant drugs may also come from studies of simpler organisms. The fruit fly, *Drosophila melanogaster*, has emerged as a promising model organism to study the molecular genetics of responsiveness to volatilized free-base cocaine. *D. melanogaster* responds to cocaine exposure with

This work was supported by Grants from NARSAD (M.S.S.), the National Institute on Drug Abuse (DA07595 and DA12408) (S.G.A.), National Institutes of Health Grant GM/DA27318 (J.H.), and the Deutsche Forschungsgemeinschaft (P.P.).

Parts of this work were presented at the 29th Annual Meeting of the Society for Neuroscience (*Soc Neurosci* 1999;**25:**160).

ABBREVIATIONS: DA, dopamine; NE, (−)-norepinephrine; 5-HT, 5-hydroxytryptamine (serotonin); DAT, dopamine transporter; SERT, serotonin transporter; NET, norepinephrine transporter; TA, *p*-tyramine; dSERT, *Drosophila melanogaster* serotonin transporter; OA, octopamine; TMD, transmembrane domain; ceDAT, *Caenorhabditis elegans* dopamine transporter; dDAT, *Drosophila melanogaster* dopamine transporter; NTT, neurotransmitter transporter; hDAT, human dopamine transporter; bp, base pair(s); kb, kilobase pair(s); EST, expressed sequence tag; RT, reverse transcriptase; PCR, polymerase chain reaction; COS-7, SV40-transformed African green monkey kidney cells; MDCK, Madin-Darby canine kidney; KRH, Krebs-Ringer's-HEPES; hSERT, human serotonin transporter; hNET, human norepinephrine transporter; EL, extracellular loop; PK-C, protein kinase C; AA, amino acid; CNS, central nervous system; Epi, (−)-epinephrine; fET, frog epinephrine transporter; TCA, tricyclic antidepressant.

many of the same stereotypic motor behaviors seen in vertebrates and also displays behavioral sensitization, as reflected in an increase in the locomotor response that develops after repeated cocaine exposures (McClung and Hirsh, 1998). DA and/or 5-HT mediate the locomotor responses to cocaine (Bainton et al., 2000; Li et al. 2000). The receptor pharmacology underlying the behavioral response to psychostimulants seems to be mediated through a quinpirole-sensitive, D2-like dopamine receptor at least in the nerve cord, but the molecular identity of the receptor remains unknown (Yellman et al., 1997). Additional studies have shown that the trace amine tyramine (TA) is required for cocaine sensitization (McClung and Hirsh, 1999) but does not stimulate nerve cord locomotor responses by itself. However, it is not clear whether TA in flies acts on postsynaptic receptors or on presynaptic transporters to produce a sensitized response. TA can activate α2-like adrenergic receptors in *D. melanogaster* (Saudou et al., 1990; Kutsukake et al., 2000), but in mammals, TA acts as an indirect sympathomimetic in that it stimulates carrier-mediated efflux of neurotransmitters by an exchange mechanism (Bönisch and Trendelenburg, 1988).

By analogy to the carriers identified in vertebrates, we reasoned that the *D. melanogaster* monoamine transporters would belong to the Na^+/Cl^--dependent symporter family (Amara and Kuhar, 1993) and would also be sensitive to cocaine. Moreover, a *D. melanogaster* cocaine-sensitive serotonin transporter, dSERT, has been identified (Corey et al., 1994; Demchyshyn et al., 1994), but the restricted expression of dSERT in serotonergic neurons implies the existence of additional high-affinity transporters to efficiently clear other monoamines such as DA, OA, and TA. All monoamine transporters cloned thus far contain a characteristic aspartate-residue in the first transmembrane domain (TMD1), which is critical for substrate transport activity (Kitayama et al., 1992). Using these criteria, we identified a putative monoamine carrier in the fly genome that displays ~65% sequence similarity with the mammalian DATs and NETs and a dopamine transporter from *Caenorhabditis elegans* (ceDAT; Jayanthi et al., 1998).

Here we report the identification, anatomical localization, and functional characterization of the *D. melanogaster* dopamine transporter, dDAT. Transport by dDAT has a pharmacological profile distinct from the DATs of mammalian species: although it retains the substrate selectivity of a dopamine carrier, dDAT is potently blocked by antidepressants and displays an inhibitory profile more similar to the mammalian NETs. The hybrid molecular features observed in dDAT and the phylogenetic analysis of vertebrate and invertebrate NTTs suggest that dDAT, along with ceDAT, represents a primordial monoamine transporter gene that existed before the emergence of the mammalian catecholamine carrier subtypes, DAT and NET. Our studies further demonstrate that dDAT is a cocaine target in *D. melanogaster* and that its functional interaction with TA in vitro could potentially link the essential role of TA in the cocaine-induced sensitization phenomenon with the modulation of dopaminergic signaling.

Experimental Procedures

Materials. All tritiated compounds used in this study were from NEN Life Science Products (Boston, MA), except [^{3}H]DA, which was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). CitalopramHBr was a kind gift from Lundbeck A/S (Copenhagen, Denmark); amphetamine isomers and RTI-55 were obtained from the NIDA Drug Supply Program. Other chemicals and drugs were purchased from Sigma-Aldrich-RBI (St. Louis, MO).

cDNA Cloning. We used the sequence information of the 1st transmembrane domain (TMD1) of hDAT to search for related sequences in the Berkeley Drosophila Genome Project database (http://www.fruitfly.org) and identified a 526-bp EST, GH22929. Translation of GH22929 revealed an essential aspartate residue (D79 in hDAT), suggesting that this EST belonged to a monoamine transporter cDNA. This EST was also part of the genomic clone AC005647, located on chromosome 2R, region 53C7-C14. Detailed analysis of the genomic sequence enabled us to identify the start and stop codons of an open reading frame of a potential monoamine transporter. We designed two oligonucleotides (5′ cgc ggt acc g tcg cag **atg** tca cca acc gga c 3′ and 5′ ccg tct aga **tca** gac atc gac ggg ttc ctt ggc g 3′) flanked by consensus sites for Asp718 and *Xba*I, respectively. A cDNA pool was generated by reverse transcription (RT-AMV, Roche Biochemicals, Nutley, NJ) of random hexamer primed total RNA isolated from *D. melanogaster* heads (Canton-S, wild type strain, a kind gift of Mike Forte, Vollum Institute) using the TRIzol reagent from Life Technologies (Gaithersburg, MD). The oligonucleotide pair was used to amplify a single 1.9 kb PCR fragment from this *D. melanogaster* head cDNA pool. The cycling program was 94°C for 3 min, 15 cycles touch down at 94°C for 45 s, 65°C (−1°C/cycle) for 30 s, 72°C for 3 min, and 25 cycles 94°C for 45 s, 55°C for 15 s, 72°C for 3 min (TaqDNA polymerase, Roche Biochemicals).

The isolated and purified DNA fragments (QIAquick; Qiagen, Chatsworth, CA) were cut with the restriction enzymes Asp718 and *Xba*I (Roche Biochemicals) and ligated into pEGFPN3 (CLONTECH, Palo Alto, CA) and pOTV (Sonders et al., 1997) using T4 DNA Ligase (Roche Biochemicals). Cutting the vector pEGFPN3 with Asp718 and *Xba*I removed the enhanced green fluorescent protein gene from the vector backbone and allowed expression of the inserted dDAT cDNA from the CMV promoter.

For comparative studies, we also cloned a dSERT cDNA fragment from the *D. melanogaster* head cDNA pool, using oligonucleotides that were designed to amplify the open reading frame of the dSERT reported by Demchyshyn et al. (1994): 5′ cgg ctc gag **atg** gac cgc agc ggg agc tcc g 3′ and 5′ cgg tct aga ctg ggc gtg gtc atg **tca** cac cg 3′. The obtained RT-PCR fragment was subcloned (*Xho*I and *Xba*I) in the same expression vectors as the respective dDAT clones (pEGFP-N3 and pOTV) and encoded the open reading frame as in accession no. U04809. All constructs were sequenced on a 373 DNA Sequencer (Applied Biosystems, Foster City, CA), using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

In Situ Hybridization. Distribution of dDAT mRNA in *D. melanogaster* third instar larval brains (Oregon-R, wild-type strain) was analyzed by in situ hybridization as described in Lehmann and Tautz (1994), with modifications. Briefly, digoxigenin-labeled antisense and sense single-stranded DNA probes were prepared by two independent single primer polymerase chain reactions against 200 ng of linearized dDAT cDNA using the primers, 5′-GCC CGT AAA CCG TGA TGA AGA GCA G-3′ for antisense and 5′-CTG GGT CAG CAC AAT CGT AAG GGT G-3′ for sense probes. Hand-dissected larval brains were fixed with 4% paraformaldehyde, treated briefly with proteinase K, and incubated with digoxigenin-labeled antisense and sense probes in parallel. After extensive washing, digoxigenated probes were detected using alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Biochemicals) and subsequent standard nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color reaction.

Expression Profile Analysis. PCR-based Drosophila RapidScan Gene Expression Panel (OriGene Technologies, Rockville, MD) was used to examine the level of dDAT mRNA in different developmental stages as well as in male and female head and body following the manufacturer's instruction. Using the primers (forward, 5′-

CCGTC GATTTA ACAAA CGTCT GG-3′; reverse, 5′-ACTGC ATAGGG AAAGA GGGCA G-3′) for dDAT, PCRs were carried out with Ampli*Taq* Gold (Perkin Elmer, Norwalk, CT) through 25 cycles of 94°C/30 s, 55°C/30 s and 72°C/1 min. The ethidium bromide-stained agarose gel was imaged on an Image Station 440CF (Kodak Digital Science, Rochester, NY) and the net intensity of bands were analyzed by 1D Image Analysis Software (Kodak Digital Science).

Tissue Cultures and Transfection Protocols. COS-7 cells (SV40-transformed African green monkey kidney cells; American Type Culture Collection, Manassas, VA), and MDCK cells (Madin-Darby canine kidney cells) were grown at 37°C in a 5% CO_2, humidified atmosphere in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal calf serum, 10 U/ml penicillin, and 10 μM/ml streptomycin. The medium for stable-transfected MDCK cell lines was supplemented with 0.5 mg/ml G418 (Life Technologies).

Transient transfection of the dDAT-pEGFPN3 construct into COS-7 cells was performed in 24-well plates, using 3 μg of dDAT-pEGFPN3 DNA and 15 μl of Fugene 6 Reagent (Roche Biochemicals) per multiwell plate, following the instructions of the manufacturer. COS-7 cells were assayed 2 days after transfection. To generate a cell line stably expressing dDAT, we used the dDAT-pEGFPN3 construct and Fugene 6 Reagent to transfect MDCK cells, which were subjected to G418 selection until individual colonies could be isolated and tested for [^{3}H]DA uptake. Of several stable cell lines, one was used for detailed analysis (dDAT-MDCK cells).

Uptake Experiments. Uptake assays were performed at room temperature in Krebs-Ringer's-HEPES (KRH) buffer (125 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, and 25 mM HEPES, pH 7.4), supplemented with 0.1% D-glucose, 1 mM ascorbic acid, 1 mM tropolone [catechol-*O*-methyltransferase (EC 2.1.1.6)-inhibitor] and 10 μM pargyline (monoamine oxidase-B inhibitor). Before the assay, cells were washed once with KRH and equilibrated for 5 min. COS-7 cells were assayed in 24-well plates and incubated for 2 min with tritiated amines, whereas MDCK cells were incubated for 6 min in 48-well plates. Nontransported inhibitors were preincubated for 5 min, and substrates were applied together with the tritiated substrate. The uptake assay was terminated with two washes of ice-cold KRH, and the accumulated radioactivity was recovered by lysing the cells in 0.2% SDS and 0.1 N NaOH and counting on a Liquid Scintillation Analyzer 1900 TR (Packard, Meriden, CT). Nonspecific uptake was determined in the presence of 20 μM nisoxetine (for dDAT and hNET), 10 μM GBR12909 (for hDAT), 20 μM fluoxetine (for hSERT), or 20 μM mazindol (for dSERT and dDAT).

Experiments to determine the ionic requirements for dDAT-mediated uptake were done in KRH buffer, substituting LiCl or choline Cl for NaCl (sodium-dependence) or substituting D-gluconates for NaCl and KCl, and $Ca(NO_3)_2$ for $CaCl_2$ (chloride dependence). Cells were washed twice with sodium- or chloride-free KRH before the assay (each wash step at least 5 min). In all transport assays, incubation periods and substrate concentrations were chosen such that uptake obeyed first-order rate kinetics.

V_{max} values for amine uptake in stable transfected dDAT-, hDAT-, and hNET-MDCK cells (Table 1) were determined in parallel assays for at least two amines per experiment and expressed as relative values, setting the V_{max} for NE = 1 to control for varying cell densities between different assays. Typically, dDAT MDCK cells, cultured in 48-well plates, accumulated DA with a maximal velocity of 80 to 200 fmol/s/well, depending on the cell density.

In Vitro Transcription, Oocyte Injection and Voltage Clamp Recording. *Xenopus laevis* oocytes were prepared as described in Sonders et al. (1997) and maintained at 17° or 21°C. cRNAs were transcribed and capped in vitro (mMessage mMachine, Ambion, Austin, TX) from pOTV plasmid containing the coding sequence of dDAT, and oocytes were tested 1 to 7 days after cRNA injection. Uptake and electrophysiological experiments were performed in a frog Ringer's buffer (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 7.5 mM HEPES/3 mM Tris, pH 7.4) at room temperature as described previously (Sonders et al., 1997). Nonspecific uptake was defined using water-injected oocytes.

Efflux Experiments. Efflux assays were performed with dDAT- and dSERT-expressing COS-7 cells 2 days after transfection. The assays were done at room temperature with the same KRH-buffer that was used for uptake assays, except that it contained 2 μM Ro 41–0960 to inhibit catechol-*O*-methyltransferase-activity, instead of tropolone. Cells were washed once with KRH-buffer and then preloaded for 10 min with 20 to 50 nM [^{3}H]amine. The preloading buffer was aspirated and cells were washed for 5 min in KRH-buffer. Efflux was initiated by replacing the wash buffer with KRH-buffer containing the cold test compounds. After 10 min, the supernatant was collected for scintillation counting (bath counts = efflux), cells were immediately washed twice with ice-cold KRH-buffer and subsequently lysed with 0.2% SDS, 0.1N NaOH, and the remaining radioactivity was counted (cell counts). Efflux (bath counts) was expressed as percent of the total recovered tritium (bath counts + cell counts) for each condition done in duplicates. Spontaneous (nonspecific) release of tritium was determined in parallel by incubating preloaded cells with KRH-buffer alone (vehicle) and was slightly lower than efflux measured in the presence of saturating concentrations of the high-affinity uptake inhibitor, mazindol (Fig. 7). The slight stimulating effect of mazindol reflects inhibition of transporter-mediated clearance of spontaneous released [^{3}H]amine. Time-course experiments showed that DA-stimulated efflux reaches a plateau level after 5 to 10 min, whereas spontaneous efflux hardly changes after 5 min (data not shown). Therefore, an efflux time of 10 min was chosen to obtain robust efflux over vehicle control levels, even for poor substrates.

Data Analysis. The results of liquid scintillation counting were used to calculate the [^{3}H]amine uptake into cells, expressed as femtomoles per milligram per well. In each experiment, the mean result from triplicate wells for each treatment was used. Specific uptake was calculated as the difference between uptake of [^{3}H]amine in the absence (total uptake) and presence (nonspecific uptake) of 10 to 20 μM high-affinity transport inhibitor for each plate. K_T and IC_{50} values were calculated from nonlinear regression analysis of the

TABLE 1

Analysis of transport kinetics for the *D. melanogaster* and human catecholamine transporters

Uptake of [^{3}H]amine was determined in stable transfected MDCK cells (dDAT-MDCK, hNET-MDCK, and GFPhDAT-MDCK) as described under *Experimental Procedures*. K_T values are presented as means ± S.E.M. from three to five experiments. V_{max} values for different substrates for a given transporter were determined in parallel assays to compensate for differences in cell densities. V_{max} values and the ratio V_{max}/K_T were normalized for NE = 1.

	dDAT			hDAT			hNET		
	K_T	V_{max}	V_{max}/K_T	K_T	V_{max}	V_{max}/K_T	K_T	V_{max}	V_{max}/K_T
	μM			μM			μM		
DA	4.8 ± 0.4	1.4	16	3.7 ± 1.1	1.2	9.1	0.7 ± 0.2	0.8	2.6
NE	55 ± 9	1	1	28 ± 7	1	1	2.3 ± 0.3	1	1
TA	61 ± 3	0.6	0.5	6.5 ± 0.9	0.4	1.7	0.3 ± 0.1	0.3	2.3
Epi	47[a]	0.4	0.5						

[a] Mean of n = 2 experiments.

data for each individual experiment according to Michaelis-Menten kinetics. All uptake data were analyzed using GraphPad Prism 2c software (GraphPad Software, San Diego, CA).

Amino acid and nucleic acid sequence alignments and bootstrapping were done with CLUSTAL W and CLUSTALTREE software (Thompson et al., 1994), which were accessed from the Biology WorkBench (http://workbench.sdsc.edu/) or were part of MacVector software (Oxford Molecular Group). Subsequently, the sequence alignment data were translated into an unrooted phylogenetic tree using DRAWTREE software, which is part of the PHYLIP 3.5c package (Felsenstein, 1989) and also available from Biology Workbench. Hydrophobicity analysis was done with GREASE (Biology WorkBench) and MacVector software.

Results

Identification of a *D. melanogaster* Monoamine Transporter cDNA. Using the sequence information of the first transmembrane domain (TMD1) of the human dopamine transporter (hDAT), we identified a *D. melanogaster* gene for a potential monoamine transporter on chromosome 2R in the Berkeley fly genome database (accession no. AC005647) (Fig. 1). PCR primers complementary to the predicted first and last exons of the open reading frame were used to amplify a single 1.9-kb DNA fragment from a *D. melanogaster* head cDNA pool. The isolated cDNA encoded a polypeptide of 631 amino acids with highest homology to the human monoamine transporters NET, DAT, and SERT and the *C. elegans* DAT (52, 49, 45, and 51% identity, respectively; Fig. 2), and was classified as the *D. melanogaster* dopamine transporter (dDAT), because of its restricted expression in dopaminergic cells and the functional properties outlined below. Sequence and hydrophobicity analyses of the carrier protein predicted 12 TMDs, intracellularly located N- and C-termini, and a large extracellular loop (EL2) between TMD3 and TMD4 (Fig. 1B). The highest degree of similarity to the mammalian catecholamine carriers is found in the 12 TMDs (~80% to both hNET and hDAT), whereas the amino- and carboxyl-terminal tails and the large EL2 show significantly less homology (≤50%).

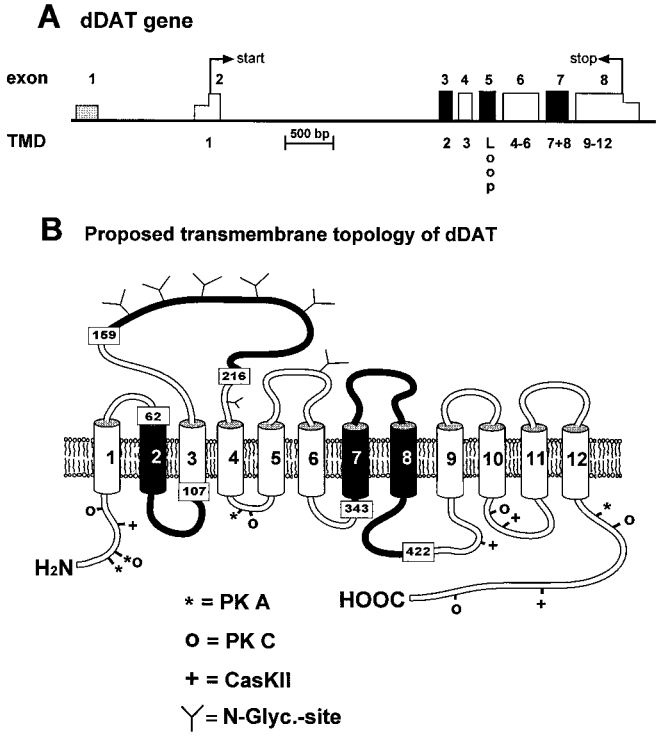

Fig. 1. The dDAT gene. A, exon-intron structure of the dDAT gene. Eight exons are shown as boxes, introns are the lines between the boxes, drawn approximately to scale. The first 5′-untranslated exon is indicated as gray box. Coding exons are shown as alternating white and black boxes that correspond to domains in the dDAT protein shown below in B. B, transmembrane topology of dDAT as predicted from hydrophobicity analysis. Domains that are encoded by sequential exons are drawn alternating white and black. Numbers at the exon borders indicate the respective amino acid residues. Location of intracellular consensus-sites for protein-phosphorylation are shown: T12, T14, S259, and S581 (*, R/KXXS/T for cAMP-dependent kinases); T14, S31, S261, T500, S585, and T624 (○, S/TXR/K for PK-C); and S24, S434, S504, and T611 (+, S/TXE/D for casein kinase II). Y, potential *N*-glycosylation sites.

Within the sequence of dDAT, there are a number of potentially significant protein motifs. A leucine zipper-type repeat observed in TMD2 of several members of the NTT family (Amara and Kuhar, 1993) is also found in dDAT (AA66 to AA87). Interestingly a second such zipper-motif is present in TMD5 (AA270 to AA291). Multiple consensus sites for phosphorylation by protein kinase C, casein kinase II, and cAMP-dependent kinases are located in intracellular domains of dDAT (Fig. 1B). Noteworthy are three serine residues (S261, S504, and S581) conserved among the *D. melanogaster* and human catecholamine transporters, which are part of PK-C, casein kinase II, and cAMP-dependent kinase consensus sites, respectively. Additionally, the *D. melanogaster* and human DATs share two PK-C consensus sites (S585 and T624) located in the C-terminal tail, a region with otherwise only moderate sequence homology. The presence of seven potential *N*-glycosylation sites, of which six are found in the large extracellular loop (EL2) and one in EL3, suggests that dDAT is a glycoprotein as has been demonstrated for its mammalian homologs. Two highly conserved cysteine residues in EL2, which were shown to be critical for the functional expression and membrane trafficking of the monoamine transporters (Chen et al., 1997) are also present in dDAT (C148 and C157).

The nucleic acid sequence of the cloned dDAT cDNA (accession no. AF260833) had nine silent mutations and one conservative AA exchange (H16 to R) compared with the genomic sequence. In addition to the dDAT cDNA isolated by RT-PCR, we independently cloned a dDAT cDNA by screening a *D. melanogaster* embryonic cDNA library (S. K. Park and J. Hirsh, unpublished observations). Both clones show the same open reading frame of 631 AA, but the library-derived clone lacks the H16-to-R mutation. Side-by-side analysis in transiently transfected COS-7 cells resulted in virtually identical DA-uptake kinetics (K_m and V_{max}) and sensitivities against mazindol, cocaine, nisoxetine, desipramine, fluoxetine, GBR12909 and (+)-amphetamine (data not shown), demonstrating that the conservative H16-to-R mutation does not have a measurable effect on transporter function. BLAST searches of the nearly complete *D. melanogaster* genome failed to detect additional monoamine transporter candidates in the same family other than the previously characterized dSERT (Corey et al., 1994; Demchyshyn et al., 1994).

The Organization of the dDAT Gene. Comparison of the isolated cDNA fragments with the genomic sequences indicated that the dDAT gene is composed of eight exons spanning 5.9 kb on chromosome 2R (Fig. 1A). In contrast, the human DAT and NET genes are composed of 14 coding exons

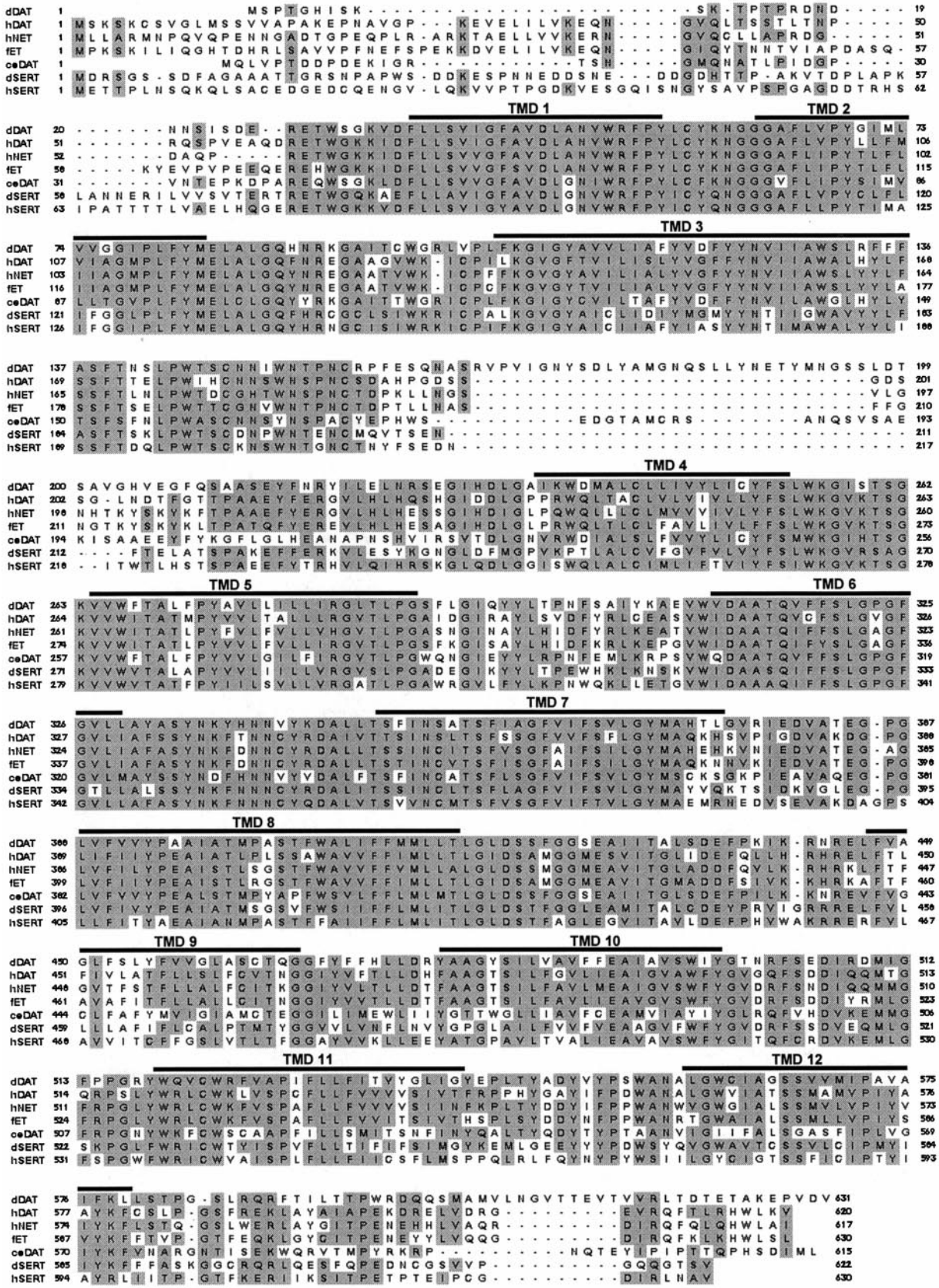

Fig. 2. Amino acid alignment of monoamine transporters. Alignment was carried out with CLUSTAL W using MacVector software. Conserved and similar residues (present in ≥4 transporters) are shaded. Putative TMDs as predicted by hydrophobicity analysis are indicated by the black bars drawn above the sequences. The accession numbers of the aligned sequences are: ceDAT (AF79899), dDAT (AF260833), dSERT (U04809), fET (U72877), hDAT (NP_001035.1), hNET (NP_001034.1), and hSERT (NP_001036.1).

(Kawarai et al., 1997; Pörzgen et al., 1995). The locations of introns within the *D. melanogaster* and human monoamine transporter genes are conserved across species with the sole exception of an additional intron in the dDAT sequence encoding the large EL2 (at proline 159). Exon 5, which encodes most of this extracellular loop, contains five of the seven predicted *N*-glycosylation sites and displays only modest homology to other mammalian transporter exons. Interestingly, the algorithms used by the Drosophila Genome Project to predict coding sequences did not identify this particular region of the gene as an exon, highlighting the importance of comparing results from in silico and in vitro approaches. The dDAT gene contained only short introns (60–70 bp), except for the first two introns, which are 0.9 and 2.4 kb in size. One striking feature of the dDAT gene is that the large intron 2 shows islands of remarkable homology (up to 45% nucleic acid identity) to regions in the corresponding hDAT intron (data not shown), implying a strong evolutionary pressure to conserve the sequence and function of this intron. Interestingly, this intron has been implicated in cell-type specific expression of hDAT (Kouzmenko et al., 1997). In addition, we used two RT-PCR based strategies to investigate whether alternatively spliced dDAT cDNAs exist. Both approaches were successfully used to identify alternative splicing of the human norepinephrine transporter gene (Pörzgen et al. 1998). The first strategy used gene-specific primers designed to amplify the predicted ORF to detect alternative splicing of internal exons. The second approach used a gene-specific sense primer and an oligo-dT18-adapter primer to identify alternative C-terminal exons. After sequencing 35 dDAT cDNA clones, we did not find any evidence for alternative splicing of the dDAT gene.

Temporal and Spatial Expression of dDAT. Although the monoamines DA, OA, and 5-HT are widely distributed in the *D. melanogaster* CNS, immunocytochemical methods have identified specific neuronal cell groups, each synthesizing one of the three transmitters (Budnik and White, 1988; Valles and White, 1988; Monastirioti et al., 1995). We used in situ hybridization in whole-mount *D. melanogaster* third instar larval CNS to demonstrate that the distribution of the dDAT mRNA was expressed in a pattern consistent with that of DA-containing cell bodies. In the larval brain (Fig. 3B), the dDAT mRNA-probe reacts with the three DA cell groups that are found in each lobe. Figure 3C shows the characteristic staining in the ventral ganglion of the eight unpaired abdominal medial DA neurons along the midline of the nerve cord and the two rows of seven dorsal lateral DA neurons. This pattern of expression is consistent with the pattern of DA cells bodies, but is not consistent with the distribution of either serotonin or octopamine cell bodies (Valles and White, 1988; Monastirioti et al., 1996). The in situ data presented here strongly suggest that the DA system in *D. melanogaster* possesses a unique mechanism for DA clearance that is not shared by 5-HT- or OA-containing cells. We also determined the developmental profile of dDAT mRNA expression (Fig. 4). Semiquantitative RT-PCR analysis showed robust dDAT expression in late embryos (12–24 h) and during larval development. dDAT mRNA expression decreases during pupal development and increases again in adult flies, with heads showing a greatly enhanced signal over bodies. The low level of dDAT RNA expression in 0- to 4-h embryos may be attributable to residual, maternal RNA.

Functional Expression of dDAT. To investigate the functional properties of the transporter encoded by the dDAT cDNA, we established a Madin-Darby canine kidney cell line stably expressing dDAT (dDAT-MDCK cells). In these cells, the uptake of monoamines obeyed Michaelis-Menten kinetics and was effectively blocked by nisoxetine (Fig. 5A). The rank order for the maximal uptake velocities (V_{max}) for the biogenic amines was DA > NE > TA ≥ Epi. DA was clearly the preferred substrate of dDAT because it exhibits the highest V_{max} and the highest apparent transport affinity (K_T) of any substrate examined (Table 1). dDAT-expressing cells did not show specific, saturable transport of 5-HT, indicating that 5-HT is not a substrate for dDAT (data not shown). To estimate the transport efficacies (Bönisch, 1998) for the biogenic amines DA, NE, and TA, we determined the ratio V_{max}/K_T and compared these values with data obtained from MDCK cells stably transfected with hNET and hDAT (Nguyen and Amara, 1996; Daniels and Amara, 1999). The data, as summarized in Table 1, show that the transport kinetics and the estimated substrate efficacies of dDAT are more similar to hDAT than hNET.

A hallmark of the uptake process mediated by members of this transporter family is their dependence on extracellular sodium and chloride ions (Amara and Kuhar, 1993). DA uptake into dDAT-MDCK cells was dependent on the presence of extracellular sodium and chloride ions. Complete replacement of extracellular sodium ions by equimolar concentrations of lithium or choline decreased DA uptake in dDAT-MDCK cells by ≥95%, demonstrating dDAT's strong dependence on extracellular sodium ions. Within the physiological range of sodium concentrations (up to 150 mM NaCl) DA uptake did not saturate (data not shown). In contrast, the substitution of gluconate for chloride-supported dDAT-mediated uptake at ~25% of the control value (120 mM NaCl) (Fig. 5B). Thus, the dependence of dDAT on chloride ions is not absolute and is similar to the relaxed chloride-dependence also noted with dSERT (Demchyshyn et al., 1994).

Transporter-Mediated Conductances. Because substrate uptake by monoamine neurotransmitter transporters is an electrogenic process (Mager et al., 1994; Galli et al., 1995; Sonders et al., 1997), we also evaluated the capability of various amines to elicit transport-associated currents. dDAT cRNA was injected into *X. laevis* oocytes and drug-mediated currents were recorded using a two-electrode voltage clamp protocol as described under *Experimental Procedures*. The amines were applied at concentrations approximately 5- to 10-fold higher than their observed IC_{50} values (Table 2) to ensure sufficient occupancy of the transporter. Consistent with the results of the uptake experiments in dDAT-MDCK cells, DA, NE, and TA all generated transport-associated currents in oocytes clamped at −60 mV (Fig. 5C). However, the magnitude of the TA-elicited currents was larger than would be predicted by the results of radiotracer flux studies in dDAT-MDCK cells. This discrepancy implies either that the charge/substrate flux ratio for TA uptake exceeds that for DA uptake, or that the maximum uptake velocity for each substrate varies with cellular background.

The insect neuromodulator OA, at concentrations between 300 μM and 1500 μM, elicited transport currents of significantly smaller amplitude than DA, NE, or TA (Fig. 5C), suggesting that OA is a poor substrate for dDAT. This substrate selectivity is noteworthy because OA has structural

features in common with NE (the β-OH group on the ethylamine side chain) and with *p*-tyramine (the lack of a 3-OH group on the phenyl ring), and suggests that dDAT recognizes both the presence of the β-OH on the ethylamine side chain and the absence of the 3-OH group of the catechol ring to discriminate against OA. Consistent with the current measurements, OA was an inefficient inhibitor of [^{3}H]DA uptake into dDAT-MDCK cells (IC_{50} = 281 μM), an observation that clearly contrasts with the 100-fold higher inhibitory potency of OA in hNET-MDCK cells (IC_{50} = 2.3 μM versus [^{3}H]NE) (Table 2).

Serotonin (300 μM) did not elicit a significant inward current in dDAT-expressing oocytes, a finding that agrees with the lack of 5-HT uptake observed in radiotracer flux experiments in dDAT-MDCK cells. However, the potency of 5-HT for inhibiting DA uptake (IC_{50} = 43 μM) in dDAT-MDCK cells is comparable with the potency of NE or TA (IC_{50} = 49 and 60 μM, respectively) (Table 2), suggesting that 5-HT acts as a nontransported inhibitor for dDAT (Fig. 5C).

Further analysis of the electrogenic properties of dDAT using a voltage-jump protocol revealed that there are qualitative differences in the currents associated with dDAT compared with hDAT and other mammalian DATs (Sonders et al., 1997; M. S. Sonders, unpublished observations). As illustrated by the current-voltage plots in Fig. 6, DA elicited robust transport-associated currents at negative potentials in both dDAT- and hDAT-expressing oocytes. At positive potentials, the *D. melanogaster* and human transporters display distinct electrophysiological properties: DA does not seem to block a leak conductance in dDAT as evidenced by the lack of a downward inflection of the current-voltage curve. Similarly, the nontransported inhibitor cocaine does not seem to block a leak conductance in dDAT, although it clearly blocks a constitutive conductance in hDAT (Fig. 6). Similar results were recorded with the substrates NE and TA and the nontransported inhibitor nisoxetine (data not shown).

Inhibitor and Substrate Pharmacology of dDAT. Initial radiotracer flux studies in dDAT expressing *X. laevis* oocytes and COS-7 cells suggested that transport of DA is

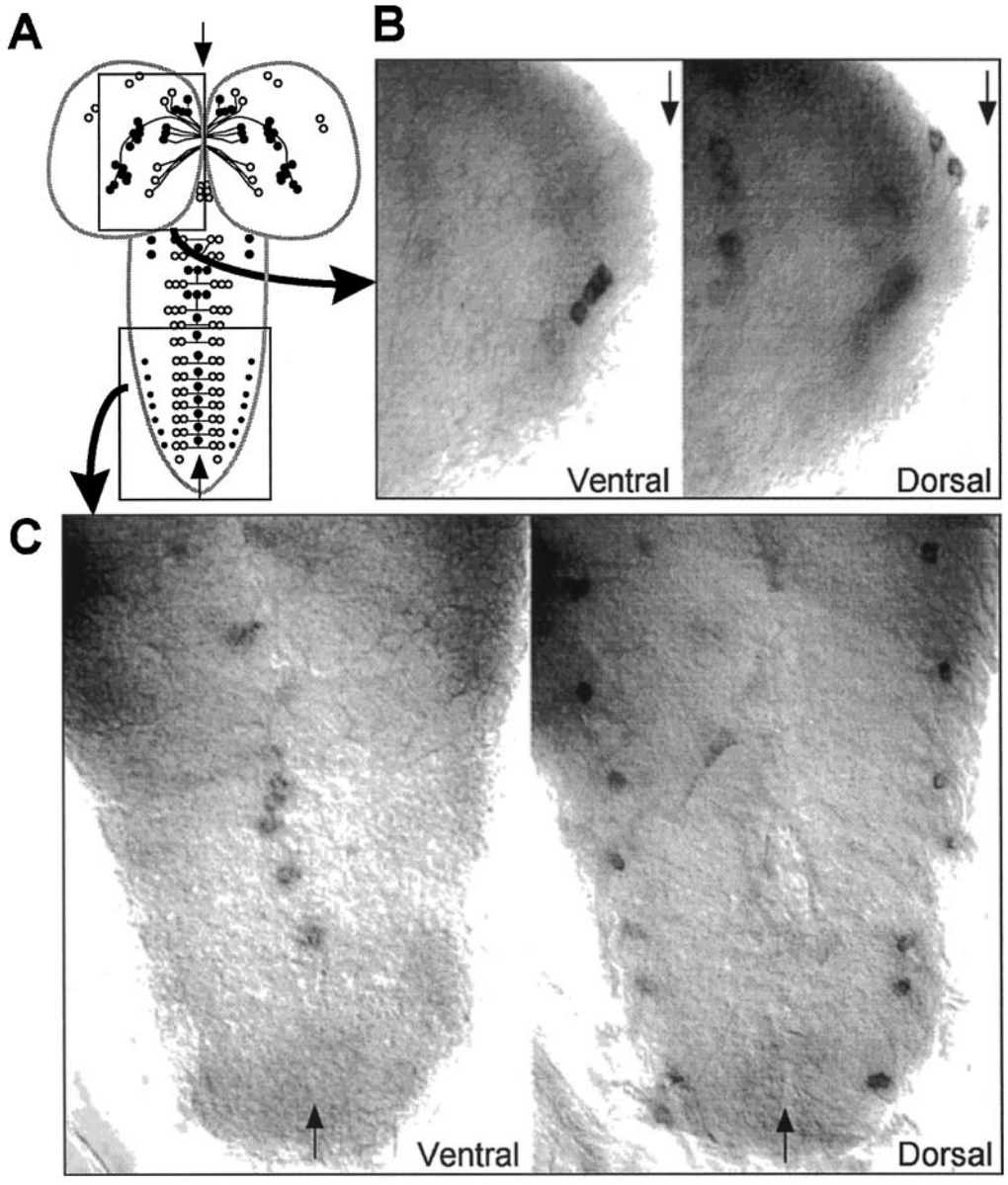

Fig. 3. Restricted expression of dDAT mRNA in dopaminergic cell bodies in *D. melanogaster* third instar larvae. A, schematic map of DA (●) and 5-HT (○) cell bodies in fly larval brain. Ventral and dorsal image of dDAT expressing cells in the brain lobe (B) and in the nerve cord (C). Arrows indicate midline.

most sensitive to the NET-selective ligands nisoxetine and desipramine but relatively insensitive to GBR 12909, a selective inhibitor of the mammalian DATs (Tatsumi et al., 1997). This similarity of dDAT to the mammalian NETs was surprising, because *D. melanogaster* do not have significant amounts of NE and because dDAT mRNA was not detected in cell bodies that contain OA, the arthropod equivalent of NE. To further explore the unexpected finding, we studied the pharmacological sensitivity (IC_{50} values) of [^{3}H]DA uptake in dDAT-MDCK cells and compared these values with data obtained for mammalian monoamine transporters (Table 2). Data for the mammalian carriers were generated using stably transfected MDCK cell lines or by using previously reported values.

Results obtained with a series of 12 high-affinity nonsubstrate inhibitors for the monoamine transporters revealed a rank order of potencies for dDAT that was clearly distinct from hDAT but most similar to the reported profiles of mammalian NETs (Table 2). Most remarkable was dDAT's high affinity for the tricyclic antidepressants desipramine, imipramine, and amitriptyline, and the NET-selective compound nisoxetine. Among the tricyclic antidepressants, desipramine shows a 150- to 300-fold selectivity for inhibiting NETs relative to SERTs (Table 2; Richelson and Pfenning, 1984; Tatsumi et al., 1997), and thus further emphasizes dDAT's NET-like inhibitor pharmacology. In contrast, the DAT-selective GBR-compounds (GBR 12909 and 12935) were at least 30 times less potent against dDAT than they are against the mammalian DATs. In addition, we determined the inhibitory potencies of a number of potential substrates of dDAT. In these experiments, dDAT displayed a 5-fold higher apparent affinity for (+)-amphetamine than for (−)-amphetamine, which is characteristic for the mammalian DATs and SERTs, but is not observed for the mammalian NETs (Richelson and Pfenning, 1984). Similarly, the biogenic amines Epi and OA and the NET substrates bretylium and guanethidine all showed a fairly low apparent affinity (IC_{50} > 100 μM) for dDAT and rat DAT (Table 2). Nevertheless, these compounds

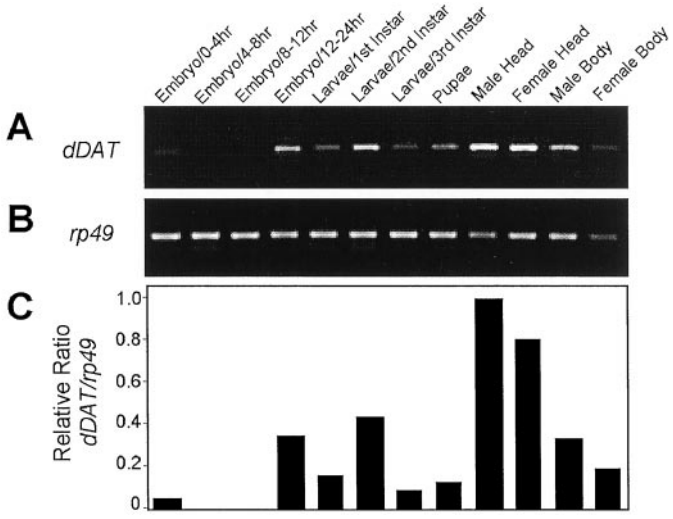

Fig. 4. Developmental regulation of dDAT expression. A, agarose gel electrophoresis of a dDAT. B, a rp49 (ribosomal protein 49) PCR-product obtained after 25 cycles with a Drosophila Rapid-Scan Gene Expression Panel (details described under *Experimental Procedures*). C, densitometric analysis of A and B: Relative level of dDAT expression compared with rp49 (normalized to dDAT/rp49 of male head = 1).

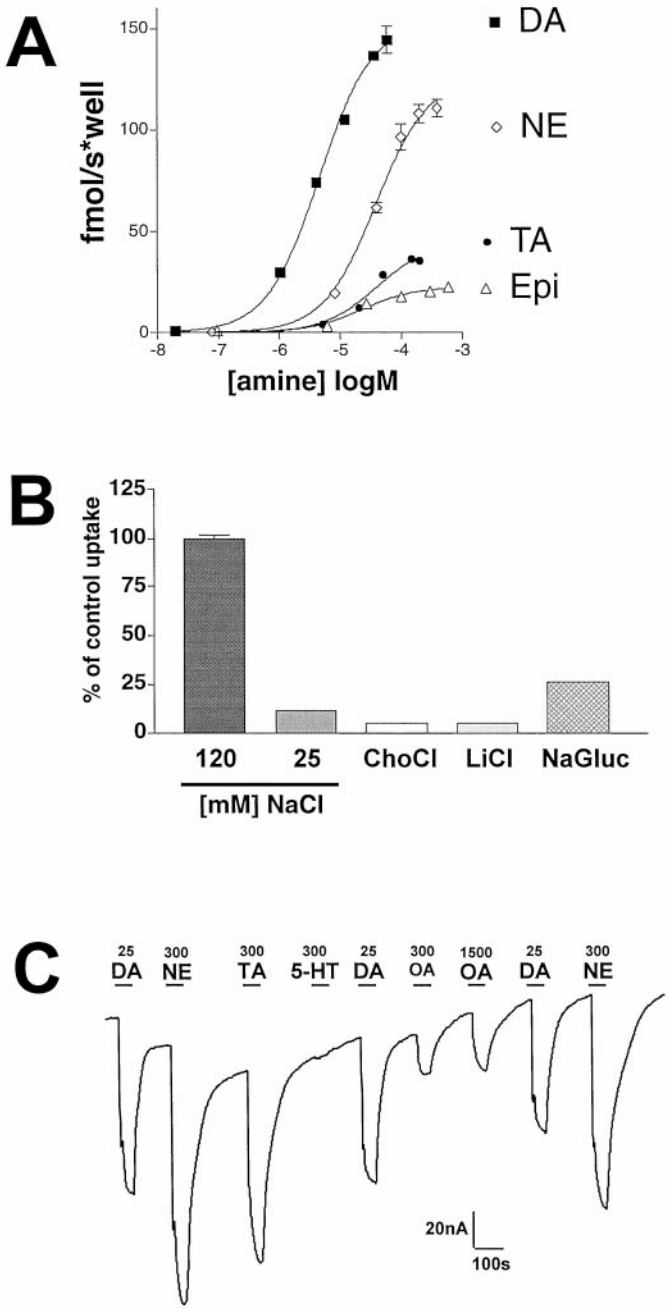

Fig. 5. Functional characterization of dDAT. A, saturation kinetics of specific amine uptake into stable dDAT-MDCK cells. Cells were assayed with 50 nM ([^{3}H]DA), 100 nM ([^{3}H]NE) or 200 nM ([^{3}H]TA or [^{3}H]Epi) and increasing concentrations of cold amine as described under *Experimental Procedures*. Nonspecific uptake was determined in the presence of 20 μM nisoxetine. Shown is a representative set of data from 2 to 5 independent experiments for each amine. The K_T and V_{max} values for [^{3}H]DA, [^{3}H]NE, [^{3}H]TA, and [^{3}H]Epi were 4.6 μM and 153 fmol/s · well; 53 μM and 107 fmol/s · well; 39 μM and 45 fmol/s · well; and 22 μM and 23 fmol/s · well, respectively. B, ion-dependence of DA uptake into dDAT-MDCK cells. Uptake assays were performed with 25 nM [^{3}H]DA. DA transport was dependent on extracellular sodium. Equimolar replacement of sodium by choline (ChoCl) or lithium (LiCl) abolished >95% of the nisoxetine-sensitive uptake. Extracellular chloride only facilitated [^{3}H]DA uptake, but was not required because complete replacement of chloride by gluconate/nitrate (NaGluc) maintained 25 to 30% of the control uptake. C, transport-elicited currents in voltage-clamped *X. laevis* oocytes, injected with dDAT-cRNA. Oocytes were clamped at −60 mV; amine concentrations are given in micromolar above each drug application and were 5- to 10-fold of their apparent affinities, as determined in dDAT-MDCK cells (see Table 2).

are readily transported by mammalian NETs (Table 2; Langeloh et al., 1987). Overall, pharmacological analysis characterizes dDAT as a unique catecholamine transporter with the substrate selectivity of the mammalian DATs, but a sensitivity to nonsubstrate inhibitors most similar to mammalian NETs.

Transporter-Mediated Efflux. The most salient function of the different monoamine carriers is the sodium-dependent, high-affinity uptake of their cognate substrates. However, monoamine transporters can also catalyze the outward transport (efflux) of intracellular amines (Bönisch and Trendelenburg, 1988). For instance, a variety of pharmacological agents that are carrier substrates, including amphetamines and TA, produce their psychostimulant and sympathomimetic effects by inducing transporter-mediated efflux of catecholamines. Because the potency of a drug to induce transporter-mediated efflux correlates with its apparent affinity to be transported, efflux studies can also provide indirect evidence whether a nonlabeled solute is a substrate for the transporter under investigation (Langeloh et al., 1987). In flies, TA has been shown to be involved in the development of sensitization to repeated cocaine exposures (McClung and Hirsh, 1999), but the mechanism for the increase in behavioral responses has not been established. A mechanism by which TA could act on dopaminergic or serotoninergic pathways and contribute to behavioral sensitization could be through the stimulation of transporter-mediated efflux of neurotransmitters. To address this possibility, we examined

TABLE 2

Pharmacological characterization of dDAT and comparison with other monoamine transporters

IC_{50} values were calculated from percent inhibition of specific [^{3}H]amine (20–100 nM) uptake into stably transfected MDCK cell lines (dDAT-MDCK, rDAT-MDCK, hNET-MDCK, and GFPhSERT-MDCK) or were taken from the literature (K_i values). Data are means ± S.E.M. from three to seven independent experiments. K_i values for competitive inhibitors should closely approximate the observed IC_{50} values because [^{3}H]amines were employed at concentrations below 10% of their respective K_T values.

	dDAT	NET	DAT	SERT	ceDAT[d]	dSERT[e]
Mazindol	4.4 ± 2.2	0.45[a]	8.1[a]	39[a]	8	3.9
Nisoxetine	5.6 ± 2.2	1.3[b]	510[b]	310[b]	3	5,000[f]
Desipramine	18 ± 5	0.9[b]	5,200[b]	340[b]	3	580
Paroxetine	22 ± 4	40[a]	490[a]	0.13[a]		3.4
Imipramine	30 ± 10	13[b]	5110[b]	42[b]	1	1450
Amitriptyline	30 ± 1	24[b]	2,300[b]	66[b]		
RTI-55	66 ± 10					444
Fluoxetine	240 ± 60	280[b]	1,600[b]	12[b]		73
GBR12909/12935	670 ± 120	310[a]	27.2[a]	940[a]	170	
Cocaine	2,660 ± 230	155[b]	270[b]	180[b]	5,000	464
Citalopram	8,100 ± 2,100	4,000[b]	28,000[b]	1.3[b]		88
Bupropion	48,000 ± 5,000	2,300[b]	630[b]	15,600[b]		
			rDAT[c]	GFPhSERT[c]		
Dopamine	2,900 ± 500	330 ± 130	4,400	118,400	146	
Norepinephrine	49,000 ± 9,000	1,470 ± 270	30,000		1,180	
m-Tyramine	23,000 ± 3,000		4,000			
p-Tyramine	60,000 ± 6,000	690 ± 180	7,500		827	
S(+)-*d*-Amphetamine	6,600 ± 900	50[b]	600	54,700	3,300	5,000[f]
R(−)-*I*-Amphetamine	34,000 ± 2,000	90[b]	4,500	266,000	13,000	30,000[f]
Epinephrine	113,000 ± 6,000	15,500[g]	200,000		3,400	
Serotonin	43,000 ± 7,000		50,000	1,560	>100,000	490
(+/−)-Octopamine	281,000 ± 27,000	2,300 ± 500	154,000		67,000	
Bretylium	175,000	14,000[g]	>100,000			
Guanethidine	535,000	8,500[g]	>100,000			
Histamine	>500,000					

[a] Tatsumi et al., 1997 [equilibrium dissociation constants (K_d) for displacing ^{3}H-ligand binding].
[b] Richelson and Pfenning, 1984.
[c] M.S. Sonders, manuscript in preparation.
[d] Jayanthi et al., 1998.
[e] Barker and Blakely, 1998.
[f] dSERT, transiently transfected in COS-7 cells, mean of $n = 2$.
[g] Mean of $n = 2$.

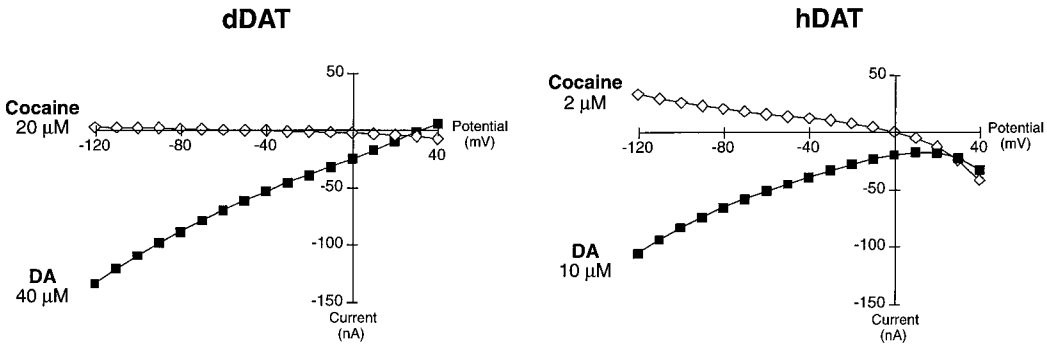

Fig. 6. Voltage-dependence of steady-state currents in dDAT- and hDAT-expressing *X. leavis* oocytes. Currents were recorded from voltage-clamped oocytes before, during, and after drug application and are represented as those elicited by drugs ($I_{drug} - I_{control}$). At negative potentials DA elicits robust inward currents in both hDAT- and dDAT-expressing oocytes. In contrast, dDAT does not mediate the pronounced ligand-sensitive proton leak conductance that is found in hDAT-expressing oocytes (Sonders et al., 1997). Block of the leak conductance is manifested in these substractions as regions of negative slope of the current-voltage curves.

the ability of TA and other bioactive amines to stimulate outward transport of preloaded [^{3}H]DA or [^{3}H]5-HT through dDAT or dSERT, respectively. COS-7 cells transiently expressing dDAT or dSERT were loaded with their respective tritiated substrates and subsequently superfused with cold amine substrates. The amount of radiolabeled neurotransmitter released during uptake of test substrate was expressed as a fraction of the total recovered radioactivity. Consistent with the idea that DA, TA and (+)-amphetamine are substrates for the carrier, these compounds stimulated efflux of preloaded [^{3}H]DA from dDAT expressing COS-7 cells, whereas 5-HT did not stimulate outward transport (Fig. 7). OA stimulated a small but significant amount of efflux, consistent with its limited efficiency in generating transport-associated currents and its low apparent affinity for dDAT. In similar experiments using dSERT-expressing COS-7 cells, 5-HT, TA, and (+)-amphetamine all induced significant efflux of preloaded [^{3}H]5-HT. However, somewhat unexpectedly, DA and, to a lesser extent, OA, stimulated dSERT-mediated efflux, suggesting that substrate recognition by dSERT is less selective than that of dDAT. These results provide additional evidence that DA and TA, but not 5-HT and OA, are efficient substrates for dDAT and support the idea that transporter-mediated efflux through dDAT and dSERT could contribute to the neuromodulatory actions of TA on behavioral responses in flies.

Phylogenetic Relationships between dDAT and Other Neurotransmitter Carriers. Because the sequence homology and pharmacological profile of dDAT overlaps with both mammalian catecholamine transporters, NET and DAT, we considered the possibility that the dDAT gene could represent an ancestral precursor of the two mammalian carrier subtypes. Thus, we explored the evolutionary relationship of dDAT within the NTT family. Phylogenetic analysis by Lill and Nelson (1998) has shown that the mammalian monoamine transporters resemble a distinct subfamily within the NTT family and can be separated into three branches: SERTs, DATs, and NETs. By constructing an evolutionary tree using nucleic acid sequence alignments of the invertebrate carriers dDAT, dSERT, ceDAT, and the mammalian biogenic amine and amino acid carrier sequences, we found that dSERT was grouped in a branch together with its mammalian homologs, rSERT and hSERT. In contrast, dDAT and ceDAT formed a separate branch that originated from the main line before the branch point for the vertebrate catecholamine carriers, DAT and NET (including the frog epinephrine transporter, fET, Apparsundaram et al., 1997) (Fig. 8). Alignments were also performed with complete and partial amino acid sequences (composed of individual exons or TMDs); in the resulting phylogenetic trees, the branching point of dDAT was placed consistently before the divergence of the vertebrate catecholamine transporters. In contrast, dSERT was predominantly found grouped in the same branch with hSERT (data not shown). These results support the assumption that the invertebrate dopamine transporters (dDAT and ceDAT) represent a primordial catecholamine transporter gene, which is also consistent with the observed hybrid NET- and DAT-like pharmacology of the two transporters (this report; Jayanthi et al., 1998).

Discussion

We report here the isolation and characterization of the *D. melanogaster* dopamine transporter gene, which was identified in the fly genome based on its sequence similarity to the human DAT. Because of its comparable sequence homology to the mammalian catecholamine transporters (NET and DAT) and the dopamine carrier from *C. elegans* (Jayanthi et al., 1998), it seemed equally possible that the identified gene could encode either a fly octopamine or dopamine carrier or a nonselective transporter for both monoamines. We applied several functional and anatomical methods to classify the isolated transporter as the *D. melanogaster* dopamine transporter. Kinetic analysis of [^{3}H]amine uptake in dDAT-MDCK cells demonstrated that DA is the preferred substrate for dDAT as reflected by the rank order of the biogenic amines for their apparent transport affinities (K_T) and transport efficacies (V_{max}/K_T), DA > NE ≥ TA = Epi (Table 1). Low micromolar concentrations of DA elicit transport-associated currents in dDAT-expressing oocytes (Fig. 5c and 6) and also effectively stimulate dDAT-mediated efflux of preloaded [^{3}H]amines from transfected cells (Fig. 7). Furthermore, dDAT is stereoselective for (+) over (−) amphetamines and discriminates against the NET substrates bretylium, guanethidine, and OA (Table 2). Overall, the substrate selectivity of dDAT is very similar to the selectivity observed for

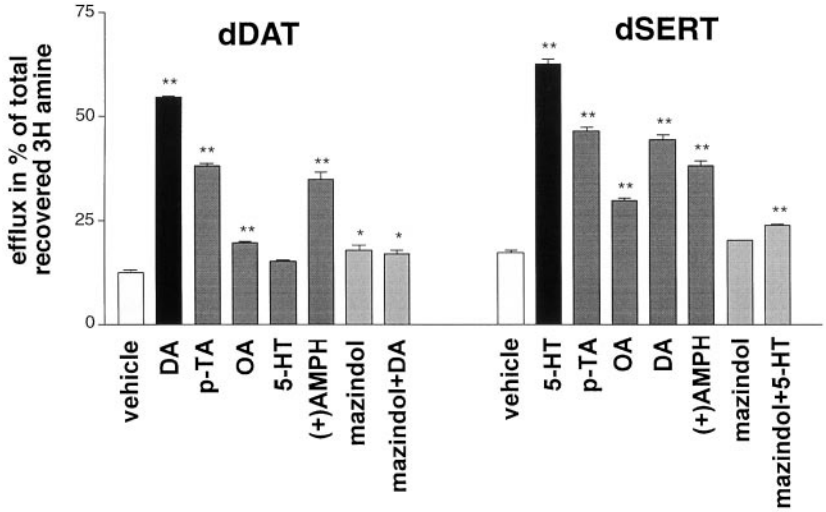

Fig. 7. Stimulation of transporter-mediated ^{3}H-amine efflux. dDAT and dSERT-transfected COS-7 cells were loaded with [^{3}H]DA or [^{3}H]5HT as described under *Experimental Procedures*. Transporter-mediated efflux was stimulated by addition of cold amine to the extracellular medium. Amine/drug concentrations were: DA, 5-HT (at their cognate carrier), and (+)-amphetamine, 30 μM; TA, 300 μM; OA, 1 mM; mazindol, 20 μM; DA (at dSERT), 300 μM; 5-HT (at dDAT), 300 μM. The amount of efflux is expressed as percentage of the total recovered ^{3}H tracer per well. Spontaneous efflux (vehicle) was determined in parallel in dDAT or dSERT-transfected cells incubated without any drug present. Data points are means ± S.E.M. from two pooled independent experiments, each performed in duplicate. Data were analyzed using one-way ANOVA analysis, followed by Tukey's multiple comparison tests, comparing all drug treatments for a given transporter with each other. P-values are shown only for the comparison of vehicle versus drug-stimulated efflux. **P <0.001; *, P <0.05.

mammalian DATs. Compatible with the functional data, in situ hybridization analysis showed that dDAT mRNA expression is restricted to DA-producing cell bodies in the fly nervous system (Fig. 3).

Inhibitor Pharmacology. The pharmacology of dDAT is complex; however, a surprising aspect of the functional properties of dDAT is an inhibitory profile resembling that of the mammalian NETs (Table 2). This trend was also described for dDATs invertebrate homolog, ceDAT. Because nisoxetine and the tricyclic antidepressants (TCAs) desipramine, imipramine, and amitriptyline have such high affinities for dDAT, its pharmacological profile can be classified as NET-like and is clearly distinct from the mammalian DATs and SERTs as well as dSERT. We therefore reasoned that the amino acid sequences of dDAT and ceDAT could provide valuable sequence information to map the contact sites of high-affinity antidepressant binding. For example, amino acid residues conserved in dDAT, ceDAT, and the mammalian NETs, but different in the mammalian DATs, could potentially contribute to the formation of an antidepressant binding site. By comparing the various sequences, we identified several AA residues that matched these criteria and were located in TMD 4 to 8, a region that had been implicated in high-affinity antidepressant binding using chimeras of the mammalian DATs and NETs (Giros et al., 1994; Buck and Amara, 1995). Another region has also been implicated in tricyclic antidepressant binding to the serotonin carriers: studies with cross-species chimeras of human and rat SERTs (Barker et al., 1994; Barker and Blakely, 1996) have identified a phenylalanine in TMD12 that confers the higher affinity for imipramine observed for hSERT compared with its rat homolog. Interestingly, the analogous AA exchange introduced in hNET does not affect antidepressant affinity. These studies suggest that multiple domains and contact sites contribute to high-affinity antidepressant binding in a transporter-specific manner. Definition of the different amino acid residues that interact with antidepressant compounds may also guide modeling studies of transporter helix packing in the absence of higher resolution structural data.

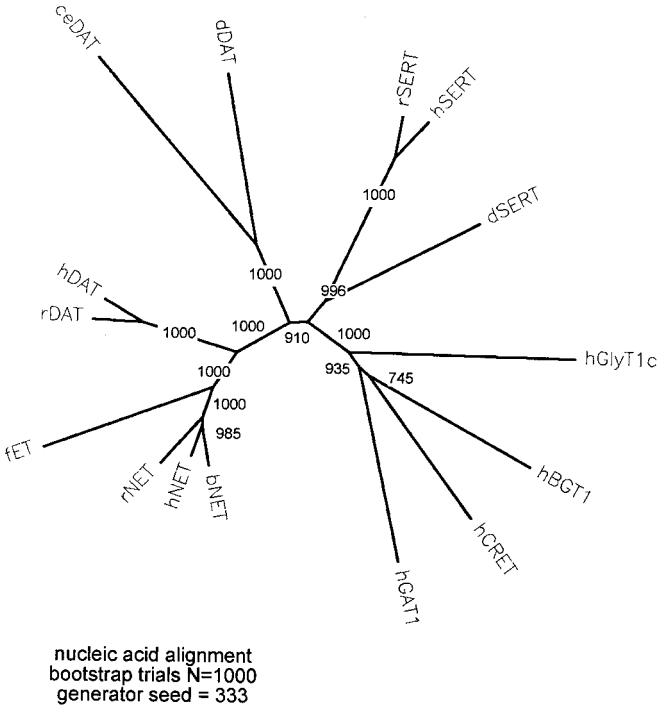

Fig. 8. Phylogenetic relationships of dDAT. Nucleic acid sequence alignment and bootstrapping of members of the Na/Cl-dependent NTT family was done with CLUSTAL W and CLUSTALTREE software, respectively (Thompson et al., 1994). Bootstrapping is a method to derive confidence values for the groupings of the tree and were calculated from $n = 1000$ independent trials with a generator seed = 333. The bootstrap values that were generated are noted for each branch. The unrooted phylogenetic tree was constructed by DRAWTREE (Felsenstein, 1989). The vertebrate catecholamine transporters (NET, DAT and fET) and the serotonin transporters (including dSERT) were grouped in two separate branches of the phylogenetic tree. The origin of a third branch containing dDAT and ceDAT is placed between these two branches, suggesting that dDAT and ceDAT originated from a gene that represents a common ancestor of both vertebrate DATs and NETs. The accession numbers of the aligned sequences are: bNET (X79015), ceDAT (AF79899), dDAT (AF260833), dSERT (U02296), fET (U72877), human betaine/γ-aminobutyric acid transporter subtype 1 (hBGT1) (L42300), human creatine transporter (hCRET; L31409), hDAT (L24178), human γ-aminobutyric acid transporter subtype 1(hGAT1; X54673), human glycine transporter c (hGlyT1c; S70612), hNET (M65105), hSERT (L05568), rat DAT (rDAT; M80233), rat NET (rNET; Y13223), rat SERT (rSERT; Y11024).

Electrophysiologial Properties of the dDAT. Both dDAT and hDAT mediate transport-associated inward currents when expressed in *X. laevis* oocytes (Fig. 5C and 6). However, in dDAT-expressing oocytes, we were unable to detect the pronounced, substrate- and inhibitor-sensitive constitutive leak conductance, which is a characteristic feature of the mammalian monoamine carriers (Mager et al., 1994; Galli et al., 1995; Sonders et al., 1997). This could mean either that dDAT does not share the proton-selective leak conductance of the human DAT or that ligand-binding does not impede a leak associated with the dDAT. In any case, dDAT seems to be a unique template to dissect mechanistic and structural aspects of these transporter-mediated conductances.

dDAT Is a Target for Cocaine. Along with dSERT (Corey et al., 1994; Demchyshyn et al., 1994), dDAT is one of two cocaine-sensitive targets identified in flies thus far. Although, in the same expression system (transiently transfected COS-7 cells), cocaine displayed a 10-fold lower apparent affinity for dDAT than for dSERT (data not shown), cocaine's low micromolar affinity for dDAT ($IC_{50} = 2.6\ \mu M$) is potentially sufficient to effectively block the transporter in vivo under the experimental conditions of exposure to volatilized free base cocaine (McClung and Hirsh, 1998). Besides, cocaine could have a higher affinity for dDAT in vivo than for the cloned transporter in vitro, a phenomenon observed for the rat dopamine transporter (Richelson and Pfenning, 1984; Kilty et al., 1991).

Significance of dDAT to Neurotransmitter Actions in Flies. Although DA is the preferred substrate for dDAT, our studies also show that the biogenic amine TA is an efficient substrate for the carrier and thus dDAT could modulate the actions of TA in the fly. TA serves as the metabolic precursor of the neurotransmitter OA, and there also seems to be a subset of neurons that selectively contains TA but not OA (J. Hirsh, unpublished observations), consistent with a possible function of TA as a neurotransmitter/modulator. Furthermore, TA acts on α2-like adrenergic receptors in insects (Arakawa et al., 1990; Saudou et al., 1990; Kutsukake et al., 2000) and, in flies, mediates physiological effects that are clearly distinct from the actions of DA and OA. For example, TA is essential for the sensitization of *D. melanogaster* toward cocaine, and a single cocaine exposure induces TA biosynthe-

sis in flies (McClung and Hirsh, 1999). However, TA does not directly stimulate the locomotor activity of decapitated fly nerve cord preparations as observed after DA, 5-HT, or OA treatment (Yellman et al., 1997). Thus, TA could act on either or both presynaptic transporters or postsynaptic receptors to modulate the behavioral sensitization observed after repeated cocaine administration.

Several observations indicate that TA could act on dDAT and dSERT. TA is transported by dDAT into transfected cell lines (Fig. 5A), it elicits transport-associated currents in dDAT-expressing oocytes (Fig. 5C), and it stimulates carrier-mediated release of preloaded substrates from dDAT- or dSERT-expressing cells (Fig. 7). Although TA and DA act in distinct ways to modulate the behavior of cocaine-treated flies, the cocaine-sensitive dDAT may be a pivotal link between the actions of TA and the dopaminergic system. TA could use dDAT and dSERT as a clearing mechanism in vivo or it could modulate DA or 5-HT signaling in the fly CNS by facilitating transporter-mediated efflux. The physiological implications for an interaction of TA and dDAT in vivo can now be further addressed using pharmacological or genetic disruption of dDAT function in flies.

High-Affinity Octopamine Clearance Does not Seem to Be Mediated by dDAT. In contrast to TA and NE, OA did not seem to be a good substrate for dDAT, based on its low apparent affinity (IC_{50} = 281 μM) for inhibiting DA uptake, the small transport-associated currents in dDAT-expressing oocytes (Fig. 5C), and its poor ability to stimulate efflux of [^{3}H]DA from dDAT-expressing COS-7 cells (Fig. 7). Moreover, we did not detect dDAT expression in octopaminergic cells, suggesting the existence of an alternative clearance mechanism for OA. Even though the genome of *D. melanogaster* is nearly completely sequenced, we were not able to identify any additional, putative monoamine transporter candidate in the GADFLY database (http://hedgehog.lbl.gov:8000/cgi-bin/annot/query), raising the possibility that OA clearance in insects is accomplished by a different transporter or metabolizing enzyme. Removal of OA by less-selective transporters for cationic amino acids (Sloan and Mager, 1999) or organic cations (Gründemann et al., 1994) could provide a plausible alternative mechanism for inactivation.

dDAT May Be a Primordial Catecholamine Transporter. Both dDAT and ceDAT share with the mammalian SERTs their high affinity for TCAs, the capability to discriminate between (+)- and (−)-amphetamines, and a moderate sensitivity for cocaine (Table 2), suggesting that these features are mediated by the same structural correlates and that these structures may represent old motifs of the monoamine transporter family. It seems unlikely that these complex features have emerged independently within the NTT gene family. Curiously, the mammalian NETs lack the selectivity to discriminate between the stereoisomers of amphetamine but are sensitive to TCAs, whereas the opposite situation is found for the mammalian DATs. Because the functional profile of dDAT has characteristics resembling those of both mammalian NETs and DATs, and the fly genome does not seem to contain an additional monoamine transporter, it seems conceivable that the dDAT gene represents a common ancestral gene for the vertebrate catecholamine transporters. Consistent with this idea, phylogenetic analysis of members of the Na^+/Cl^--dependent NTT family show the invertebrate carriers dDAT and ceDAT grouped together in a branch that emerges from the main line before the vertebrate catecholamine carriers branch into DAT and NET families, whereas dSERT is clearly grouped together with its mammalian SERT homologs. Consequently, we propose that the genomes of such invertebrates as *D. melanogaster* and *C. elegans* contain only two distinct monoamine transporter genes, a SERT and a catecholamine transporter. The emergence of the distinct, vertebrate DAT and NET families may be the consequence of a gene duplication event of a primordial catecholamine transporter gene that is still unique in the invertebrate genomes of *D. melanogaster* and *C. elegans*.

Acknowledgments

We thank the Berkeley *Drosophila* Genome Project for sequence information and their public availability (http://www.fruitfly.org). Debra Peth and Yuqin Yang contributed outstanding technical assistance to this project. We are indebted to Drs. Rebecca P. Seal, Balakrishna Prasad, and Ole V. Mortensen for valuable contributions and comments during the course of this work, and to Mike A. Forte for a generous supply of *D. melanogaster* heads. We are particularly grateful to Holger Lill (Universität Osnabrück, Germany) for his helpful comments on the phylogenetic data.

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