A genetic screen in *Caenorhabditis elegans* for dopamine neuron insensitivity to 6-hydroxydopamine identifies dopamine transporter mutants impacting transporter biosynthesis and trafficking

Richard Nass,*<sup>†</sup> Maureen K. Hahn,<sup>†</sup> Tammy Jessen,<sup>†‡</sup> Paul W. McDonald,<sup>‡</sup> Lucia Carvelli<sup>†‡</sup> and Randy D. Blakely<sup>†‡</sup>

Departments of *Anesthesiology and †Pharmacology, ‡Center for Molecular Neuroscience, †Graduate Program in Neuroscience, Vanderbilt School of Medicine, Nashville, Tennessee, USA

Abstract

The presynaptic dopamine (DA) transporter (DAT) is a major determinant of synaptic DA inactivation, an important target for psychostimulants including cocaine and amphetamine, and a mediator of DA neuron vulnerability to the neurotoxins 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium ion. To exploit genetic approaches for the study of DATs and neural degeneration, we exploited the visibility of green fluorescent protein (GFP)-tagged DA neurons in transgenic nematodes to implement a forward genetic screen for suppressors of 6-OHDA sensitivity. In our initial effort, we identified three novel *dat-1* alleles conferring 6-OHDA resistance. Two of the *dat-1* alleles derive from point mutations in conserved glycine residues (G55, G90) in contiguous DAT-1 transmembrane domains (TM1 and TM2, respectively), whereas the third allele results in altered translation of the transporter’s COOH terminus. Our studies reveal biosynthetic, trafficking and functional defects in the DAT-1 mutants, exhibited both in vitro and in vivo. These studies validate a forward genetic approach to the isolation of DA neuron-specific toxin suppressors and point to critical contributions of the mutated residues, as well as elements of the DAT-1 COOH terminus, to functional expression of catecholamine transporters in neurons.

**Keywords:** *Caenorhabditis elegans*, dopamine, genetics, 6-hydroxydopamine, nematode, transport.


Dopamine (DA) plays an essential modulatory role in the vertebrate central nervous system, including activity in systems supporting fine motor control, attention, cognition, and reward (Grace *et al.* 1998; Spanagel and Weiss 1999). DA transporters (DATs) are responsible for the reuptake of DA following release and thereby limit the impact of DA in space and time (Gainetdinov and Caron 2003). DATs also support non-vesicular DA release that can be triggered by amphetamines (Jones *et al.* 1998), second messengers (Gnegy *et al.* 2004; Khoshbouei *et al.* 2004) or synaptic stimuli (Falkenburger *et al.* 2001). DATs are important targets for multiple psychostimulants and also facilitate entry of neurotoxins such as 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium (Sachs and Jonsson 1975; Javitch *et al.* 1985). DATs are members of the SLC6 family of Na<sup>+</sup> and Cl<sup>−</sup> coupled, plasma membrane transporters (Kilty *et al.* 1991; Shimada *et al.* 1991; Goldberg *et al.* 2003). DATs contain 12 transmembrane domains (TMs) with a large, N-glycosylated extracellular loop between TMs 3 and 4, and intracellular NH2 and COOH termini. DATs form higher order complexes following the application of cross-linkers (Hastrup *et al.* 2001; Sitte *et al.* 2004), suggesting that functional DATs form homomultimers. DATs also appear to interact with multiple membrane embedded and cytoplasmic regulatory proteins, including syntaxin 1A, PICK-1, α-synuclein and Hic-5 (Lee *et al.* 2001a; Torres *et al.* 2001; Carneiro *et al.* 2002; Lee *et al.* 2004).

Received February 1, 2005; revised manuscript received March 21, 2005; accepted March 25, 2005.

Address correspondence and reprint requests to Randy D. Blakely, PhD, Suite 7140, MRBIII, Vanderbilt School of Medicine, Nashville, TN 37232–8548, USA. E-mail: randy.blakely@vanderbilt.edu

**Abbreviations used:** DA, dopamine; DAT, dopamine transporter; GFP, green fluorescent protein; HA, hemagglutinin; NET, norepinephrine transporter; 6-OHDA, 6-hydroxydopamine; SERT, serotonin transporter; TM, transmembrane domain.
DATs are conserved across phylogeny, with functionally validated orthologs evident in vertebrates and invertebrates. For example, DATs have been cloned and characterized from *Caenorhabditis elegans* (Jayanthi et al. 1998), *Drosophila melanogaster* (Porzgen et al. 2001), and *Trichoplusia ni* (Gallant et al. 2003), with significant identity evident in comparison with rodent and human DAT proteins. *Caenorhabditis elegans* DAT (DAT-1) exhibits 45% amino acid identity with the human ortholog and possesses many functional similarities, including saturable, high affinity Na+ and Cl− dependent transport (Km = 1.2 µmol/L) and sensitivity to D-amphetamine, cocaine, GBR 12909 and nomifensine. In addition, DAT-1 activity, assayed both in transfected cells (Jayanthi et al. 1998) and in primary *C. elegans* cell cultures (Carvelli et al. 2004), is sensitive to low nanomolar concentrations of the tricyclic antidepressant imipramine. Using transgenic expression of a *dat-l* promoter fusion to green fluorescent protein (GFP) (Nass et al. 2002), we provided evidence for the exclusive expression of DAT-1 in *C. elegans* DA neurons (CEP, ADE, PDE). Additionally, we documented the sensitivity of *C. elegans* CEP and ADE neurons in vivo to exogenous application of 6-OHDA, toxicity abolished by co-incubations of 6-OHDA with DAT blockers as well as by genetic disruption of the *dat-l* gene. Here we describe implementation of a forward genetic screen for suppression of DA neuron 6-OHDA sensitivity and our recovery of multiple *dat-l* alleles that alter the sequences of highly conserved DAT amino acid residues. We find that these mutations significantly impact DAT localization and function in vitro and in vivo and lead to a loss of DAT-mediated DA transport activity. Additionally, our studies provide validation of a forward genetic approach for the identification of functional alleles of neurotransmitter transporters.

**Methods**

*Caenorhabditis elegans* strains and maintenance

*Caenorhabditis elegans* strains were cultured on bacterial lawns of either OP-50 or NA-22 at 20°C according to standard methods (Brenner 1974; Hope 1999). N2 Bristol is the wild-type strain. The BY200 vtls1 [Pdat-1::GFP; rol-6] and BY250 vtls7 [Pdat-1::GFP] strains are integrated, transgenic lines expressing GFP from the *dat-1* promoter has been previously described (Nass et al. 2002).

Genetic screen for dopamine transporter mutants

Standard Ethyl Methanesulfonate (EMS) mutagenesis (Brenner 1974) was performed on BY200 hermaphrodites. Embryos (F3) obtained from the F2 generation (approximately 2000 genomes) were isolated by hypochlorite treatment of gravid adults as previously described (Hope 1999). After 17–24 h incubation in M9 buffer to obtain synchronized L1s, the worms were washed once in 10 mL dH2O, spread on 8P/NA22 plates and incubated at 22°C for 25–29 h. L3 larvae were collected, washed in dH2O, and added to the assay mix (to a final OD600 worms of 0.1–0.2) with 10 mM ascorbic acid and 50 mM 6-OHDA (Sigma, St Louis, MO, USA). The assay mix was incubated for 1 h at 22°C and mixed gently every 15 min. The worms were then washed in dH2O and spread on NGM/OP50 plates. Approximately 60 h later, animals that retained GFP in all four cephalic sensilla (CEP) dendrites were transferred onto separate NGM plates and allowed to produce progeny (F4). Synchronized L3 larvae from these animals were collected as described above and the DNA was isolated from animals that maintained GFP fluorescence to search for evidence of mutations within the *dat-l* gene. Animals (F5) isolated from the initial screen as fully insensitive were retested as described above to confirm lack of 6-OHDA effects.

*Caenorhabditis elegans* genomic DNA isolation

Genomic DNA was isolated from animals washed in dH2O and pelleted at 2000 g for 2 min. After washing in dH2O, volumes were adjusted to 1 mL with 0.1 M Tris (pH 8.0) and the Tris/worm slurry was dripped into weigh boats containing liquid N2 using a sterile transfer pipette. The liquid nitrogen was allowed to evaporate and frozen worms were transferred to a polypropylene 15-mL conical tube, adding an equal volume of worm lysis buffer (100 mM Tris pH 8.0, 10 mM NaCl, 50 mM EDTA pH 7.4, 1% sodium dodecyl sulfate) in addition to 10 µL of proteinase K (20 mg/mL). Samples were incubated at 67°C for 40 min and then heated to 95°C for 30 min. After addition of 5 µL RNAse A (10 mg/mL), samples were incubated at 37°C for 30 min, and extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v) three times, followed by overnight ammonium acetate precipitation at −80°C. EtOH (80%)-washed pellets were resuspended in 400 µL of Tris/EDTA and analyzed on a 1% agarose gel prior to PCR.

Identification of *dat-l* mutations

Genomic DNA from wild-type (N2), BY200 and 6-OHDA resistant lines was used as a template for PCR of the *dat-l* gene and subsequent dieoxyxynucleotide DNA sequencing. The Expand Long Template PCR kit (Roche Diagnostics, Indianapolis, IN, USA) was used to amplify a 3.8-kb fragment consisting of 5′-UTR through to the 3′-UTR, past the *dat-l* polyadenylation site. A 3.8-kb fragment was gel purified using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA, USA). The fragments were sequenced using sense and anti-sense overlapping *dat-l* specific primers spanning the entire gene with Big Dye Terminator Cycle Sequencing Mix (ABI, Foster City, CA, USA) and analyzed on an ABI 310 Sequencer (Center for Molecular Neuroscience Neurogenomics Core). When point mutations were identified, the PCR amplification from genomic DNA was repeated in duplicate. Both reactions were sequenced separately to confirm the point mutation and rule out random polymerase error.

Reverse transcription–polymerase chain reaction from total *Caenorhabditis elegans* RNA

One mutant recovered in the screen exhibited a 10 bp deletion at bp1819 (AF079899) accompanied by a 172 bp insertion at the beginning of exon 12, which normally encodes much of the transporter’s C-terminus. To examine the impact of this deletion on the expressed mRNA and protein cDNA from this mutant (hereafter termed dat-lΔ K584R for the first residue bearing altered translation in the ectopic COOH-terminus), was prepared from total RNA using
variant DAT-1 constructs were confirmed by DNA sequencing. HA-tagged dat-1/pcDNA3 vector, yielding RB665. Sequences of Gel Extraction kit) and ligated into WI, USA). A 400 bp fragment containing the altered DAT-1 cloned into the pGEM-T Easy Vector System I (Promega, Madison, D in both the DAT-1/pcDNA3 and HA-tagged DAT-1/pcDNA3 expression vectors described above to create the

Construction of DAT-1/pcDNA3 and HA-DAT-1/pcDNA3 expression vectors

Construction of the hemagglutinin (HA)-tagged DAT-1 cDNA in pcDNA3 (RB606) for mammalian cell expression studies was performed using the Quick Change Site-Directed Kit (Stratagene, La Jolla, CA, USA). Two 51 base pair primers of the same sequence (sense and anti-sense) were annealed to the translation start site of the dat-1 gene with the 27 bp HA sequence inserted directly after the ATG. Confirmation of the insert and integrity of the cDNA sequence were verified by DNA sequencing.

Construction of G55E, G90E, and DAT-1A K584R mutations in DAT-1/pcDNA3 and HA-DAT-1/pcDNA3 expression vectors

Oligonucleotide-mediated site-directed mutagenesis was used as described above to create the dat-1 G55E and G90E point mutations in both the DAT-1/pcDNA3 and HA-tagged DAT-1/pcDNA3 expression plasmids. To produce expression constructs for ΔK584R mutant, the 1.9kb fragment of dat-1 ΔK584R cDNA amplified from the RT–PCR total cDNA product was initially TA cloned into the pGEM-T Easy Vector System I (Promega, Madison, WI, USA). A 400 bp fragment containing the altered DAT-1 sequence was excised with SacII and NorI, gel purified (QIAquick Gel Extraction kit) and ligated into SacII/NorI digested DAT-1/pcDNA3 (RB454) yielding plasmid RB664, as well as into the HA-tagged dat-1/pcDNA3 vector, yielding RB665. Sequences of variant DAT-1 constructs were confirmed by DNA sequencing.

Construction of dat-1 mutants in a Caenorhabditis elegans expression vector

To examine the impact of mutations of DAT-1 protein expression in vivo, the G55E and G90E point mutations were introduced into a Pdat-1::GFP-DAT-1 translational fusion, whose construction is detailed elsewhere (Carvelli et al. 2004), creating mutant constructs RB805 and RB806, respectively. The ΔK584R mutant was made using the Advantage 2 PCR kit (BD Biosciences, San Jose, CA, USA) to amplify a 400 bp region from the ΔK584R DAT-1/pcDNA3 plasmid, simultaneously adding a BglII site on the 3' end of the product for convenient cloning to create expression construct RB807. Mutations were confirmed by DNA sequencing.

Dopamine transport assays in transfected COS-7 cells

COS-7 cells were plated at 100 000 cells per well in 24-well tissue culture plates (Perkin Elmer, Boston, MA, USA) with 500 μL of DMEM (Invitrogen, Carlsbad, CA, USA) complete medium [10% fetal bovine serum (HyClone, Logan, UT, USA), 100 U/mL Penicillin, 100 μg/mL Streptomycin, and 2 mM L-Glutamine]. After 24 h at 37°C and 5% CO2, the cells were transfected with 200 ng of DAT-1 expression vector DNAs (mutant or wild-type DAT-1, as either HA-tagged or untagged constructs) using 0.6 μL of FuGENE 6 transfection reagent (Roche Diagnostics). Cells were incubated further with transfection reagent plus DNA for 48 h. Cells were washed once with 37°C Krebs’–Ringer’s–HEPES buffer including 10 mM d-glucose, 100 μM ascorbic acid, 100 μM paraglyine, and 1 mM tropolone. Cells were pre-incubated at 37°C in the same buffer for 10 min. Some wells contained the DAT-1 antagonist imipramine (1 μM final) to assess non-specific uptake. All cells were washed for 10 min at 37°C with 50 mM of [3H]DA (3,4-[3H]dihydroxyphenyl)ethylamine, Perkin Elmer) and then washed three times with ice-cold Krebs’–Ringer’s–HEPES buffer. Counts accumulated were solubilized using Microsrt 20 (Perkin-Elmer) scintillation fluid with rocking for 1 h before quantitation using a Top Count scintillation counter (Packard, Meriden, CT, USA). DAT saturation kinetics (10 nM–5 μM, constant DA specific activity) were examined using cells transfected with the DAT-1 G90E/pcDNA3 and DAT-1ΔK584R/pcDNA3 mammalian constructs (100 ng/well) as described above except that non-specific DA transport activity was assessed using 2 μM imipramine (final concentration).

Western blot analyses

COS-7 cells were plated at 100 000 cells per well in 24-well tissue culture plates as described above. After 24 h the cells were transfected with 200 ng of HA-tagged DAT-1 expression vector DNA (mutant or wild type) and 0.6 μL of FuGENE 6 transfection reagent per well. At 48 h post transfection, cells were washed three times with 2 mL of phosphate-buffered saline at room temperature and extracted with (100 μL) radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris, pH 7.4, 150 mM EDTA, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% sodium deoxycholate) with a 1:250 dilution of mammalian cell protease inhibitor cocktail (Sigma), shaken at 4°C for 30 min, and supernatants collected by centrifugation (13 000 g for 1 h). Protein concentrations were determined using the Coomasie Plus Protein Assay Reagent (Bio-Rad) and samples were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and transferred to 0.45 μm polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA). Blots were incubated with a rat monoclonal anti-HA-peroxidase labeled antibody [High Affinity (3F10), Roche Diagnostics] at a 1:2000 dilution for 1 h at room temperature. After washing, blots were developed with Western Lightening Chemiluminescence reagents (Perkin Elmer) and exposed to High Performance Chemiluminescence film (Amersham, Piscataway, NJ, USA).

To analyze surface expression, cells were biotinylated with sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate-(sulfo-NHS-SS-Biotin) (1.0 mg/mL; Pierce, Rockford, IL, USA) as previously described for studies of norepinephrine transporter (NET) and serotonin transporter (SERT) (Ramamoorthy and Blakely 1999; Sung et al. 2003).

Caenorhabditis elegans cell culture and transport assays

Embryonic C. elegans cells were prepared according to Christensen et al. (2002) by first treating synchronized adult nematodes with an alkaline hypochlorite solution (0.5 M NaOH and 1% NaOCl). After 5 min of treatment, eggs released were washed two times with sterile egg buffer containing: 118 mM NaCl, 48 mM KCl, 2 mM MgCl2, 25 mM HEPES and 2 mM CaCl2 (pH 7.3, 340 mOsm) and then separated from the carcasses by density centrifugation in 30%...
sucrose. The egg layer was removed by pipette and washed two times with sterile water. Eggshells were lysed with 0.5 U/mL chitinase (Sigma) dissolved in egg buffer and rocked gently for 15–30 min at room temperature. After incubation with chitinase, the suspension was gently triturated to dissociate the cells. Cells were washed with L-15 cell culture media (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (Gibco), 50 U/mL penicillin, and 50 µg/mL streptomycin (Gibco), adjusted to 345 mOsm with sucrose, and then filtered through a sterile 5-µm Durapore syringe filter (Millipore) to remove undissociated embryos and newly hatched larvae. Filtered cells (10⁶) were plated on 12-mm diameter glass coverslips coated with 0.5 mg/mL peanut lectin agglutinin (Sigma). Cultures were maintained in L-15 cell culture medium at 28°C in a humidified incubator. Two days after dissociation and plating, C. elegans embryonic cells were washed twice with buffer (130 mM NaCl, 10 mM HEPES, 1.5 mM CaCl₂, 0.5 mM MgCl₂, 1.3 mM KH₂PO₄, 34 mM dextrose and 0.1 mM ascorbic acid, tropolone and pargyline, pH 7.35) and incubated with 50 mM [³H]-DA (NEN, dihydroxyphenylethylamine) for 5 min at room temperature. Uptake was terminated after three washes of ice-cold buffer. Radioactivity from cells was extracted with 1% sodium dodecyl sulfate for 20 min. All assays were repeated in triplicate with statistical evaluation performed using Prism 4.0 (Graphpad Software, San Diego, CA, USA) on a Macintosh workstation. Specific tests are noted in figure legends.

Production and imaging of Caenorhabditis elegans transgenic lines
Stable transformants were created by co-injection of plasmid constructs with a marker plasmid using standard methods. Transgenic animals containing either a GFP:DAT-1 translational fusion [RB538 (wild-type DAT-1 sequences)], or the GFP-tagged DAT-1 mutant constructs (RB505, RB806) were obtained after co-injection of a final concentration of 15 ng/µL along with 60 ng/µL of pRF4[rol-6(su1006)], 50 ng/µL of carrier DNA (pBluescript, Stratagene) into the N2 (Bristol) strain. Transformants made using the RB807 vector were obtained after co-injection of 60 µg/µL RB807, 60 ng/µL pRF4, and 15 ng/µL pBluescript. F1 animals positive for rol-6 phenotype were then segregated and allowed to derive progeny. Plates containing >90% rol-6 animals were kept and allowed to clone for several generations to verify the stability of the extrachromosomal array. For each of the different plasmids injected (RB538, RB505, RB806, RB807), three stable lines derived from different L1 transformants, displaying a stable >90% penetrant rol-6 phenotype, were used for imaging. Transgenic animals were maintained on small OP50/NA22 plates at 20°C. At 12 h prior to imaging, animals were washed off plates using MilliQ H₂O, leaving only unhatched embryos behind. Animals were allowed to hatch and feed ad libitum on the OP50/NA22 plates during the 12 h. Well-fed L2/L3 animals were washed three times in MilliQ H₂O and then treated with a 0.01% tricaine/0.001% tetramisole (final concentration) anesthetic 10 min prior to being placed onto a microscope slide containing a 2% agarose pad. Confocal images were captured with a Zeiss LSM 510 confocal microscope (Vanderbilt Cell Imaging Shared Resource, supported by NIH grants CA68485, DK20593, DK58404, HD15052, and DK59637). All lines were imaged using a 40 x Plan-Neofluar, N.A. 1.3 oil objective. Z-series optical sections were taken at 0.5 µm with a pinhole set at 1 µm. DIC images were captured sequentially during 488 scan. DIC images used represent a single plane through the middle of the animal, whereas GFP images represent a 3D projection combining all sections. All image processing and montage assembly were performed using Adobe PHOTOSHOP.

Results
Dat-1 alleles identified as suppressors of 6-hydroxydopamine sensitivity
To identify genes mediating sensitivity of C. elegans DA neurons to 6-OHDA, we implemented a genetic screen utilizing pdat-1::GFP transgenic animals, isolating animals that retained the expression of GFP despite a 1 h exposure to 50 mM 6-OHDA. This treatment selectively diminishes DA neuron GFP fluorescence and is associated with dendritic fragmentation and cell death (Nass et al. 2002). Adult mutants that retained GFP expression in the four CEP neurons were isolated, and if 40% of their progeny maintained resistance to the neurotoxin (compared with control animals at about 8%), lines were cloned for further analysis. Of the approximately 4000 haploid genomes screened, we isolated 17 mutant lines that exhibited varying degrees of tolerance to 6-OHDA (Fig. 1).

As a dat-1 deletion mutant has been shown to display 6-OHDA resistance (Nass et al. 2002), we sought to define first whether any of our mutant lines exhibiting 6-OHDA insensitivity carried dat-1 mutations that could explain insensitivity to the toxin. We focused on five lines (Fig. 1) that appeared to be completely insensitive to the toxin, owing to the ease of tracking a fully resistant phenotype in lines derived from our initial isolates. We amplified genomic DNA

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from resistant and control (BY200) animals and compared these to public database sequences for the dat-1 gene. In these efforts, we identified dat-1 mutations in four of the five 6-OHDA resistant lines, representing three distinct coding alterations (Fig. 2). Mutant line #25 (BY298 dat-1G55E(vt2); vtIs1) possessed a single G to A mutation (GGG → GAG) that converts a highly conserved glycine at amino acid 55 in TM1 to glutamate (G55E). All described members of the SLC6A family have a Gly at this position, though residues on either side can diverge. Two separate lines (#13,#62) (BY402 dat-1G90E(vt3); vtIs1) (BY299 dat-1G90E(vt3); vtIs1) were found to bear a different G to A (GGA → GAA) mutation that converts a glycine to a glutamate at amino acid 90 in TM2 (G90E). Remarkably, this glycine residue is also highly conserved across the gene family, unlike many other residues of TM2. Examination of genomic DNA from line #52 (BY423 dat-1D582R(vt4); vtIs1) revealed a deletion/insertion at the beginning of exon 12. This mutation, confirmed by sequencing of an RT–PCR product from line #52 mRNA, shifts the reading frame at K584, to add 13 new amino acids after the end of predicted TM12 (RTDKAVTQISATFCN; D584R) rather than the 32 amino acids (KWQRVTMPYR-KRPQTEYIPITQPHSDIML) that would normally complete the DAT-1 COOH terminus. The altered splicing and termination of the DAT-1 COOH terminus in the D584R mutant actually matches an erroneous database deposit for the DAT-1 COOH terminus, derived from informatics-based translation of C. elegans transcription units (D88551, NCBI database). This sequence was subsequently corrected by our group during the dat-1 cDNA cloning effort (Jayanthi et al. 1998). Although the DAT-1 COOH terminus is not well conserved with mammalian orthologs, the new COOH terminus eliminates a homologous region that in mammalian DATs has been reported to interact with the multiple LIM domain protein Hic-5 (Carneiro et al. 2002) and also removes a conserved C-terminal Type II (Φ-X-Φ) PDZ binding motif. In mammals, this sequence in DAT has been implicated in the binding of the PDZ domain protein PICK-1 and is required for their colocalization in DA neurons (Torres et al. 2001). Finally, line #2 (BY408 vt5; vtIs1) lacked mutations within the dat-1 gene. Preliminary mapping studies indicate that the EMS-induced trait of 6-OHDA insensitivity in line #2 maps with polymorphic markers linked to a chromosome other than the one that harbors dat-1. Efforts to identify the gene targeted in this line are ongoing and will be the described in a future report.

Evaluation of in vitro phenotypes of DAT-1 mutants

Although a dat-1 deletion line confers 6-OHDA resistance (Nass et al. 2002), the new dat-1 alleles we recovered might not represent the fundamental basis for toxin resistance if, despite outcrossing, they are accompanied by closely linked mutations. To determine whether the new dat-1 alleles detrimentally impact DAT-1 protein synthesis, stability,
trafficking or function, we heterologously expressed wild-type and mutant DAT-1 cDNAs, followed by biochemical and functional studies. Using site-directed mutagenesis, we engineered the recovered alleles in both untagged and epitope (HA)-tagged DAT-1 mammalian expression constructs and transiently transfected them into COS-7 cells. Immunoblots of extracts from HA-tagged DAT-1 (wild-type) expressing cells (T in Figs 3a and b) reveal the presence of a tight band of immunoreactivity at ~50 kDa and a broad smear of bands running from 90 to 120 kDa (hereafter referred to as the 100 kDa species). Both the 50 kDa and 100 kDa bands are absent from the vector (pcDNA3) transfected cells. The electrophoretic mobility of these bands suggest the presence of both immature, core glycosylated DAT-1 protein (50 kDa species) and mature, complex N-glycosylated DAT-1 (100 kDa species), a pattern seen with mammalian DATs after heterologous expression (Hastrup et al. 2001; Li et al. 2004). In previous studies with DAT-1 related transporters, the more mature species predominates on the cell surface (Hastrup et al. 2001; Bauman and Blakely 2002; Hahn et al. 2003). Indeed, anti-HA immunoblotting of streptavidin-recovered biotinylated extracts from wild-type DAT-1 (Figs 3a and b) demonstrated a marked enrichment of the 100 kDa material in biotinylated (surface, S) fractions. Parallel analyses of the DAT-1 mutants demonstrate that each mutation has significant, yet distinct, effects on DAT-1 protein expression and/or trafficking. Representative blots are provided in Figs 3(a) and (b) with quantitative analyses of total and surface protein levels provided for both the 50 kDa and 100 kDa species in Figs 3(c) and (d). Analysis of DAT-1 G55E protein expression reveals a modest reduction of both

![Western blot analyses of wild-type and mutant dat-1 variants.](image)

(a) COS-7 cells were transfected with pcDNA3, HA-DAT-1 (control), or dat-1 variants as described in Methods. For biotinylation studies, cells were incubated with sulfo-NHS-SS-biotin 24 h post transfection followed by RIPA/protease inhibitor buffer extraction. Equal amounts of protein blotted to assess levels of total dopamine transporter 1 (DAT-1) protein. From the remaining samples, streptavidin beads were used to extract biotin-labeled dopamine transporter 1 (DAT-1) as described in the Material and Methods section. Blots were probed with a horse-radish peroxidase-conjugated monoclonal antibody recognizing the hemagglutinin (HA) tag followed by chemiluminescent detection (Amersham). (b) Same evaluation as described in (a) except the comparison is between pcDNA3, control, and the mutant variant (ΔK584R). (c) Band density of total protein expression was compared between HA-DAT-1 and each of the mutant variants. The optical density analysis was performed on both the mature (~90 kDa) and immature (~50 kDa) forms of expressed DAT-1 protein. (d) The same analysis as in (c) was performed on the streptavidin-isolated material (surface expression). Results are displayed as percentage ± SEM of HA-DAT-1 band density for each DAT-1 species with quantitation derived from at least three separate experiments. Statistical analysis was performed with one-way ANOVA followed by Dunnett’s multiple comparison test. *p < 0.05.
imature and mature protein levels in total fractions with a more striking loss of mature protein occurring in surface fractions, consistent with a trafficking deficit.

In contrast to DAT-1 G55E, DAT-1 G90E exhibits a shift in the ratio of mature to immature protein in total extracts, with immature protein predominating. Although a reduction of mature DAT-1 G90E protein was evident in surface fractions (~50% of wild-type DAT-1), this was by no means as severe as seen for the G55E mutant. Furthermore, the G90E mutant exhibits detectable quantities of immature protein in surface fractions. These findings indicate altered efficiency of biosynthetic progression from core to complex N-glycosylated forms accompanied by deficits in surface expression. Cells expressing the DAT-1 ΔK584R variant demonstrated a major loss of total DAT-1 protein, evident with both mature and immature species, and little or no material of either form was recovered in surface fractions (Figs 3b and d).

In parallel with biosynthetic and trafficking studies, we evaluated the impact of the engineered mutations on DA transport activity. As our HA-tagged DAT-1 construct exhibits a reduction (~50%) in transport activity relative to non-tagged wild-type DAT-1 vector (data not shown), we examined the functional properties of mutants in both a tagged and a non-tagged background. As shown in Fig. 4, assays at a fixed (50 nM) test concentration of [3H]DA reveal a significant elevation in DA accumulation for wild-type DAT-1 or HA-DAT-1 above vector (pCDNA3)-transfected cells. DA transport activity is reduced to levels achieved with the vector control when imipramine (1 μM), a potent inhibitor of DAT-1 (Jayanthi et al. 1998), was included in the assays. When assays were conducted with cells transfected with the DAT-1 mutants, none of the variants induced significant transport activity above background when the less efficient HA-tagged construct was used (Fig. 4a). In the non-tagged background (Fig. 4b), where wild-type DA transport activity is higher, we were able to detect specific transport activity arising from the G90E mutant, though activity was only ~25% of the wild-type cDNA. Nonetheless, this level of activity was sufficient to permit a kinetic evaluation where we could establish transport parameters responsible for loss of activity. In these studies (Table 1), we found no change in substrate $K_m$ relative to wild-type DAT-1, but rather observed an ~90% loss in transport capacity ($V_{max}$). Use of higher concentrations of substrate permitted us to detect residual activity as well for the ΔK584R mutant, with fits to this data consistent with retention of a normal DA $K_m$ but only ~6% the transport $V_{max}$ of wild type.

**Evidence of compromised expression and trafficking of DAT-1 mutants in vivo**
To examine the impact of DAT-1 mutations on transporter biosynthesis and trafficking in *in vivo*, we generated transgenic DAT-1 nematodes expressing wild-type or mutant DAT-1 proteins as NH2-terminal GFP:DAT-1 translational fusions in DAergic neurons. Animals produced expressing the wild-type DAT-1 translational fusion (BY312 vtIs6 [P*dat-1::GFP:*DAT-1: rol-6f]), displayed a readily detectable level of expression in all dopaminergic head neurons (CEP, ADE). This expression pattern is consistent with the DA neuron-specific expression of a *dat-1* transcripational fusion (BY200), described previously (Nass et al. 2002). We observed expression of GFP:DAT-1 along the plasma membrane of the CEP and ADE cell somas, out along dendrites, and in axons terminating in synapses localized to the nerve ring (see Fig. 5a–c). We also established that our GFP:DAT-1 fusion is functional in *in vivo* in that it fully restores 6-OHDA sensitivity to a (BY329 *dat-1(ok157); vtIs6; vtIs7), data not shown).

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<th>Table 1 Dopamine transporter 1 (DAT-1) saturation kinetics</th>
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Number of replicate experiments are given in parentheses.

* $p < 0.05$, Student’s *t*-test.
Examination of the expression pattern of animals expressing G55E (BY347 vtEx9 [Pdat-1::GFP:DAT-1G55E; rol-6]) revealed little effect on the global distribution of the transporter. GFP expression, however, was reduced in these lines relative to wild-type transgenics, consistent with an alteration in the overall amount of protein synthesized or retained within DA neurons (see Figs 5d and e). Lines produced expressing the G90E (BY349 vtEx11 [Pdat-1::GFP:DAT-1G90E; rol-6]) transgene exhibited both a reduction in total GFP signal as well as an altered pattern of localization. In all lines examined, mutant expression was evident in the ADE and CEP cell bodies, but was notably absent from either axonal or dendritic processes (see Figs 5f and g), consistent with a failure to transit to processes or an enhanced degradation after export. No appreciable GFP signal could be detected in lines injected with the ΔK584R (BY348 vtEx10 [Pdat-1::GFP:DAT-1ΔK584R; rol-6]) transgene when injected at the same concentration as other constructs, despite clear evidence of successful rol-6 transgene expression. When the mutant DNA concentration injected was increased over three times that used to create other lines (50 ng/μL), weak expression was evident and this signal was restricted to the ADE and CEP cell bodies (Figs 5h and i). To establish directly whether DA neurons bearing mutant DAT-1 proteins are functionally compromised for DA transport, we implemented C. elegans culture techniques (Christensen et al. 2002), and conducted in vitro [3H]DA transport assays using cells derived from wild-type (BY200) or mutant lines. In these cultures, we achieved approximately the same proportional representation of DA neurons (identified by expression of the DAT-1::GFP transcriptional fusion) as seen in the (BY200), minus the PDE neurons that are born postembryonically, after the time of our cell isolation. In separate studies (Carvelli et al. 2004) we characterized the high-affinity, Na+ and Cl– dependent DA transport activity of these cultures and documented DAT-1 dependent currents evident from patch-clamp recordings of the GFP-visualized neurons. Importantly, both DA transport and DA-triggered currents are lost with imipramine co-incubation and are absent in cells derived from dat-1 knockout [dat-1(ok1577)] animals. In the present experiments, cells cultured from the BY200 line originally utilized for 6-OHDA screening demonstrated clear evidence of imipramine (100 nm)-sensitive DA transport activity (Fig. 6). In contrast, no imipramine-sensitive DA transport activity could be measured in any of the mutant lines. Indeed, DA accumulation in these cells was essentially at levels observed.

**Fig. 5** Altered expression and localization of dopamine (DA) transporter 1 (DAT-1) mutants. (a) Schematized depiction of DA neuron wiring in Caenorhabditis elegans. One of each of the paired cell bodies are represented with circles, axonal projections into the nerve ring (gray circle) end in arrowheads, and dendritic projections are represented by lines alone. Wild-type (BY346 vtEx6 [Pdat-1:GFP:DAT-1; rol-6]) expression of GFP:DAT-1 fills all six cell bodies in the head, with accumulation at synaptic regions in the nerve ring (c, arrow). The G55E mutation leads to a decrease in overall expression, but retains cell body distribution and nerve ring accumulation (e, arrow). Expression of G90E and ΔK584R is reduced relative to WT and restricted to the cell soma. Note lack of nerve ring localization (g and i, arrows). Bar in each GFP image represents 10 μm.

**Fig. 6** Dopamine (DA) transport deficits in cultures from 6-hydroxydopamine (6-OHDA) insensitive lines. Transport activities of the BY-200 line and the mutant lines recovered in the screen carrying dat-1 mutations. Studies represent the mean values ± SEM of transport assays performed as described in Methods from at least three separate cultures of embryonic cells. Non-specific DA transport activity is defined with 100 nm imipramine and is equivalent to the transport activity observed in a dat-1 KO line (data not shown).
in cultures derived from the DAT-1 knockout line (Carvelli et al. 2004). These primary culture studies are consistent with the significant transport deficit evident on analysis of our DAT-1 mutants when tested in vitro using transfected mammalian cells and add support to the contention that alleles recovered compromise 6-OHDA insensitivity by limiting DAT-1 activity.

Discussion

We previously characterized C. elegans DA neuron sensitivity to the neurotoxin 6-OHDA (Nass et al. 2002; Nass and Blakely 2003), revealing degeneration to be cell-specific and dependent on DAT-1 expression. In a forward genetic screen of ~4000 haploid genomes, we recovered 17 lines with clear evidence for suppression of 6-OHDA DA neuron degeneration. Many of these lines were only partially resistant, displaying a range of sensitivity from moderate to full insensitivity. Given that a dat-1 deletion or incubation of wild-type lines with DAT-1 inhibitors fully blocks 6-OHDA sensitivity, we sought evidence for mutations in the dat-1 gene in our fully resistant lines. Of the five fully resistant lines, one (BY408) did not possess dat-1 mutations and maps to a different chromosome (R. Nass and T. Jessen, preliminary findings). This mutation, the subject of future studies, may target a modifier of dat-1 gene or protein expression or be a gene required for transporter surface targeting and/or full elaboration of transport function. Alternatively, the mutation may inactivate a gene required for intracellular toxin action following uptake. Of the four other 6-OHDA insensitive lines, three lines (BY298, BY402, BY299) harbored point mutations leading to non-conservative changes in DAT-1 amino acid sequence. The final line (BY423) carries a genomic insertion/deletion that disrupts translation of the transporter’s COOH terminus.

In COS-7 cells, compared with wild-type DAT-1, the G55E mutant derived from BY298 demonstrates modestly diminished levels of both mature and immature DAT-1 protein, suggesting that translation products may be less efficiently synthesized or more readily degraded. What is more striking from the COS-7 experiments is this mutant’s severe loss of surface expression. In vivo, the G55E mutation exhibits an overall diminished GFP signal relative to that found with wild-type DAT-1 when injections are performed with comparable DNA concentrations. G55E DAT-1 protein under the conditions we have used is exported to the processes though at visibly reduced levels. The fact that significant quantities of DAT-1 G55E appear to make it into the processes of DA neurons but cannot confer DA uptake in primary C. elegans cultures or 6-OHDA sensitivity in the intact worm suggests that, as in COS-7 cells, a disruption of normal surface trafficking occurs. We have developed DAT-1 directed antibodies that allow for localization of native and mutant DAT-1 protein by immunocytochemistry (McDonald and Blakely, unpublished findings), but these reagents have not proven sufficiently sensitive to verify a surface trafficking defect of G55E using native membranes and western blotting approaches.

In contrast to the G55E mutation, the G90E mutation demonstrates a shift in the ratio of mature to immature protein in COS-7 cells. Despite this effect, biotinylation studies reveal significant levels of surface-expressed protein in both mature and immature forms. Our findings therefore indicate that although the G90E variant can reach the cell surface in COS-7 cells, it clearly possesses a serious defect in DA catalytic activity. DA recognition may well be preserved, as the G90E $K_m$ for DA is essentially that of DAT-1. A loss of DA translocation following binding could arise from less efficient coupling of the transporter to ion gradients supporting DA uptake or to a conversion of DAT-1 to an ion channel state. Caenorhabditis elegans DAT-1 has been found to support channel states that can support the inward movement of ions and depolarize neurons (Carvelli et al. 2004). An understanding of whether these states support or limit DA transport is presently lacking. Indeed, patch-clamp studies using the G90E mutant expressed in COS-7 cells where surface protein can be measured may be illuminating as to the respective contributions of channels to substrate influx. In primary C. elegans cultures, however, no DA transport was evident, and in vivo GFP imaging indicates that although some cell body expression is evident, little transporter makes it out into the processes. Thus it is likely that the G90E mutation impacts biosynthetic progression, trafficking away from the cell soma and DA catalytic function for any carriers that reach the cell surface.

The most severe phenotype was evident with the AK584R mutant derived from line BY423. In COS-7 cells, small amounts of immature protein and no mature protein were evident in total extracts, and surface fractions were devoid of DAT-1 protein, in keeping with a complete loss of DA uptake activity. We could detect no specific DA transport activity in primary cultures derived from the BY423 cell line and could visualize no GFP signal in vivo in transgenic lines unless the DNA concentration used for injection was significantly increased. Even at a three times greater DNA concentration relative to other dat-1 mutants, only a few weakly visible soma were evident and no localization to processes could be visualized.

The changes in the structure of DAT-1 imposed by the recovered alleles resonate with studies examining structure/function relationships of DAT-1 mammalian orthologs and paralogs. Both the G55E and G90E mutations impose non-conservative amino acid changes on highly conserved residues with transmembrane domains. Even though greater overall conservation in the gene family is evident in the TM regions, TM1 and TM2 themselves are among the more highly conserved of these domains. TM1 has been implicated in recognition of ion and neurotransmitter substrates (Barker
Evidence from cysteine accessibility studies indicate that TM1, along with TM3, may line the substrate permeation pathway (Chen et al. 1997; Henry et al. 2003). G55 lies adjacent to a conserved aromatic (F/Y) residue that supports recognition of N-substituted tryptamines as well as recognition of multiple transporter inhibitors in SERT (Adkins et al. 2001). Approximately one helical turn upward in TM1 is a conserved D residue thought to interact with biogenic amine substrates, possibly through ion-pairing with the NH2 group of biogenic amine substrates (Kitayama et al. 1992; Barker et al. 1999). However, though the Gly residue in the equivalent position of the human serotonin transporter (Gly94) loses ~90% transport Vmax, when mutated to Cys, the SERT G54C mutant retains a normal 5-HT Km. Moreover, the Cys at this position is largely inaccessible to externally applied, membrane impermeant, methanethiosulfonate reagents. A comparable Cys scan of TM1 in DAT has not yet been published and therefore comparisons here are clearly speculative. Clearly though, the added charge of the Glu substitution could likely have additional effects on DA transport independent of surface trafficking. Regardless, the impact of G55E on overall DAT-1 biosynthesis and trafficking in vitro and in vivo suggests that the insertion of a charged side-group at a position where the small, non-polar Gly side chain is almost universally located negatively impacts transporter membrane insertion or trafficking. DAT proteins and homologous transporters lack NH2 terminal signal sequences and TMs 1 and 2 may insert as a unit after translation, providing the signal sequence needed to initiate threading of transporter TMs in the plasma membrane (Clark 1997, 1998).

Loss of DA transport activity of G90E despite significant surface expression in COS-7 cells supports a requirement for proper TM2 structure at one or more steps in the transport cycle. TM2 has generally received less attention with respect to specific functions, but recently has been implicated in DAT cocaine recognition through species scanning mutagenesis (Wu and Gu 2004). The G90 residue in DAT-1 also sits within a conserved heptad leucine/isoleucine repeat (‘leucine zipper’) that has been suggested to participate in transporter oligomerization (Torres et al. 2003; Korkhov et al. 2004). Mutation of this motif disrupts the ability of mammalian DATs [as well as related GAT1 proteins (Scholze et al. 2002; Korkhov et al. 2004)] to mature and reach the cell surface. In COS-7 cells, we did gather evidence for an intracellular accumulation of immature DAT-1 G90E protein, characteristic of DAT proteins that possess other mutations hindering oligomerization (Sitte et al. 2004). Our ability to detect surface expression of the mature form of G90E DAT-1 protein may thus reflect the high-level of expression achieved with the COS-7 model. We doubt this is fully the explanation for what appears to be a relatively normal pattern of surface expression, as the COS-7 expression system readily reports loss of biosynthetic progression/surface trafficking of other mutants such as A457P in the NE transporter that leads to the disorder Orthostatic Intolerance (Hahn et al. 2003). Regardless, the diminished function of the G90E mutation in the face of surface-expressed G90E DAT-1 protein indicates that altered TM2 structure at some level can impact DA recognition and/or transport. Mutations in mammalian DAT-1 prevent dominant-negative actions of other mutations linked to multimer formation (Torres et al. 2003) and thus the C. elegans DAT G90E mutation may have destabilized critical TM2 intra–oligomeric interactions that are needed for transport function in COS-7 cells. Our in vivo studies with GFP-tagged DAT-1 G90E further reveal a difference between heterologous expression approaches and evaluation of mutants in their native environment. In the latter context, biosynthetic progression and trafficking appear likely the primary determinants of loss of function for DAT-1 G90E, as it is for many mammalian DAT and GAT-1 TM2 mutants. In future studies, the transparent nature of the nematode may permit an assessment of G90E oligomerization status via fluorescence resonance energy transfer (FRET) and possible roles for oligomerization in neuronal transporter trafficking.

One of our insensitive alleles removes 32 amino acids from the DAT-1 COOH terminus, replacing it with an ectopic sequence unrelated to any sequences known in the gene family. Initial chimera studies indicated that the COOH terminus of biogenic amine transporters made no contributions to their substrate and antagonist interactions (Blakely et al. 1993; Buck and Amara 1994; Giros et al. 1994; Moore and Blakely 1994). More recent studies have revealed that this domain supports multiple protein–protein interactions and appears to be essential for transporter biosynthetic progression. The mammalian DAT COOH terminus has been found to interact with PICK-1 (Torres et al. 2001), α-synuclein (Lee et al. 2001b), and the LIM domain protein Hic-5 (Carneiro et al. 2002). A C. elegans α-synuclein ortholog is not evident in a BLAST search of genomic sequences, whereas multiple PICK-1 and Hic-5 related genes exist. Hic-5 may serve as a scaffold to link DAT to other modulators supporting DAT localization and/or function, and interacts with the juxtamembrane regions of the human DAT COOH terminus that are also disrupted in our mutant. PICK-1 associates with mammalian DATs at the distal PDZ recognition motif, a motif that is conserved in C. elegans DAT-1. In mammalian neurons, integrity of these sequences is required for colocalization of the two proteins (Torres et al. 2001). Recent studies suggest multiple uses for DAT PDZ recognition sequences (Bjerggaard et al. 2004), and findings with mammalian NET and GAT1 proteins reveal that intact COOH terminal sequences are essential for efficient endoplasmic reticulum to Golgi progression, possibly via the interaction of chaperone proteins (Bauman and Blakely 2002; Farhan et al. 2004). Finally, we recognize that the ectopic sequences completing the DAT-1 COOH terminus in...
the mutant may in themselves disrupt transporter biosynthesis or stability.

In summary, our findings provide critical proof of concept that a functional screen of C. elegans DA neuronal sensitivity to 6-OHDA can be used to identify function-modifyingDAT-1 alleles. Additional use of the screen may allow for the recovery of other dat-1 alleles whose functional analysis can extend current efforts to link transporter structure to function, gathered largely to date through heterologous expression studies. Finally, this system allows for the elucidation of dat-1 regulators and/or genes supporting DA neuron sensitivity to reactive oxygen species.

Acknowledgements

This work was supported by DK58212 (RN, TJ, LC and RDB), DA14917 (RDB), Pharmaceutical Research and Manufacturers of America Fellowship (RN) and F31NS046237 (PM). We gratefully acknowledge assistance of Samuel Arthur Moore, Jordan Knopper and Sonya Soverby in C. elegans husbandry and Dawn Matthis and David Miller for critical review of the manuscript.

References


Blakely R. D., Moore K. R. and Qian Y. (1993) Tails of serotonin and DA neuronal sensitivity to 6-OHDA can be used to identify function-modifying DAT-1 alleles. Additional use of the screen may allow for the recovery of other dat-1 alleles whose functional analysis can extend current efforts to link transporter structure to function, gathered largely to date through heterologous expression studies. Finally, this system allows for the elucidation of dat-1 regulators and/or genes supporting DA neuron sensitivity to reactive oxygen species.


