**Forward Genetic Analysis to Identify Determinants of Dopamine Signaling in Caenorhabditis elegans Using Swimming-Induced Paralysis**

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**ABSTRACT** Disrupted dopamine (DA) signaling is believed to contribute to the core features of multiple neuropsychiatric and neurodegenerative disorders. Essential features of DA neurotransmission are conserved in the nematode *Caenorhabditis elegans*, providing us with an opportunity to implement forward genetic approaches that may reveal novel, *in vivo* regulators of DA signaling. Previously, we identified a robust phenotype, termed Swimming-induced paralysis (Swip), that emerges in animals deficient in the plasma membrane DA transporter. Here, we report the use and quantitative analysis of Swip in the identification of mutant genes that control DA signaling. Two lines captured in our screen (vt21 and vt22) bear novel *dat-1* alleles that disrupt expression and surface trafficking of transporter proteins *in vitro* and *in vivo*. Two additional lines, vt25 and vt29, lack transporter mutations but exhibit genetic, biochemical, and behavioral phenotypes consistent with distinct perturbations of DA signaling. Our studies validate the utility of the Swip screen, demonstrate the functional relevance of DA transporter structural elements, and reveal novel genomic loci that encode regulators of DA signaling.

The catecholamine dopamine (DA) is a phylogenetically conserved neurotransmitter that in vertebrates, including humans, regulates motor and cognitive behavior. Altered DA signaling contributes to several disorders of the brain, including Parkinson disease, dystonia, attention-deficit/hyperactivity disorder, schizophrenia, and addiction (Carlsson 1993; Mazet-Robison et al. 2005; Seeman 2010; Kurian et al. 2011). DA signaling is achieved through both presynaptic and postsynaptic mechanisms that, to date, have been studied largely using biochemical, pharmacological, and reverse genetic approaches. These studies have uncovered and characterized many fundamental components that control DA signaling, such as the genes that provide for DA synthesis, release, reuptake, and response. The powerful, modulatory control exerted by DA over both cognitive and motor behavior and the incomplete understanding of the determinants of risk for DA-associated brain disorders suggest that a broader array of genes exists that controls DA signaling. The identification of these genes may benefit therefore from unbiased approaches, such as those afforded by forward genetic screens.

Among the most critical of known determinants of DA signaling, one molecule, the presynaptic DA transporter (DAT; *SLC6A3*), has special roles in controlling access of pre- and postsynaptic DA receptors to DA, in recycling DA into presynaptic terminals after release, and in maintaining levels of DA needed for sustained release (Giros et al. 1996; Torres et al. 2003). The knockout of DAT through homologous recombination in mice demonstrates an obligate role for the transporter in amphetamine and cocaine-induced hyperlocomotion, as well as DA release and clearance (Giros et al. 1996). Conversely, mice overexpressing DAT display reduced extracellular DA levels and heightened sensitivity to amphetamine (Salahpour et al. 2008). These studies also demonstrate that genetic manipulation of DAT in mice leads to changes in the expression of genes that encode pre- and postsynaptic DA receptors and neuropeptides, underscoring the...
importance of DAT as a key regulator of a broad DA signaling network (Giros et al. 1996). After the cloning of DAT cDNAs (Kilty et al. 1991; Shimada et al. 1991; Giros et al. 1992; Jayanthi et al. 1998; Brüss et al. 1999), many have explored the impact of DAT mutations using heterologous expression models in vitro (Schmitt and Reith 2010), although, as of yet, the significance in vivo of many of these findings is unknown. Recently, we and others have identified rare, functionally penetrant, mutations in DAT in subjects with attention-deficit/hyperactivity disorder and juvenile dystonia (Mazeli-Robison et al. 2008; Kurian et al. 2009), compelling a better understanding of the impact of DAT mutations and altered DAT regulatory mechanisms in vivo.

Forward genetic strategies that rely on DAT-dependent phenotypes provide a path to the identification of key DAT structural elements as well as the elucidation of novel regulators of DAT signaling. Although such methods overcome the bias of studies focused on known genes and pathways (Mohn et al. 2004), they are typically quite difficult to implement in mammals because of the time and cost associated with animal breeding, mutation mapping, and functional characterization in vivo. The nematode Caenorhabditis elegans has long been used in forward genetic screens (Brenner 1974), including identification of genes supporting DA signaling (Sulston et al. 1975; Schaefer and Kenyon 1995; Sawin et al. 2000; Chase et al. 2004; Allen et al. 2011; Wani et al. 2012). These and homology-based approaches have revealed C. elegans orthologs of mammalian genes required for DA biosynthesis, including tyrosine hydroxylase/cat-2 (Lints and Emmons 1999), GTP cyclohydrolase/cat-4 (Loer and Kenyon 1993), and amino acid decarboxylase/bas-1 (Bamford et al. 2004), the vesicular monoamine transporters/cat-1 (Duer et al. 1999), genes involved in DA response including the D1 and D2 type DA receptors dop-1/2/3/4 (Suo et al. 2003; Chase et al. 2004; Suo et al. 2004; Sugiuura et al. 2005), and the presynaptic transporter/dat-1 (Jayanthi et al. 1998).

In a previous study, we demonstrated that worms lacking functional DAT-1 (dat-1(ok157)) demonstrate a robust, DA-dependent phenotype when placed in water, termed Swimming-induced paralysis (Swip), a behavior that is dependent on activation of the postsynaptic, D2-like receptor DOP-3 (McDonald et al. 2007). Here, we describe our efforts to capitalize on the Swip phenotype to capture modulators of endogenous DA signaling using forward genetic approaches. Lines derived from this effort are subjected to conventional biochemical and pharmacological methods, as well as to a novel analytical platform that can more finely dissect features of disrupted motor function. We describe the isolation of multiple, independent mutant lines, including two (vt21 and vt22) that possess novel alleles of DAT-1. Disrupted expression and trafficking of the vt21 mutant provides the first demonstration of functional relevance in vivo of a highly conserved, structural feature of SLC6 transporters. Two additional lines, vt25 and vt29, lack dat-1 mutations, map to different genomic loci, and sustain distinct components of DA signaling.

MATERIALS AND METHODS

C. elegans strains, husbandry, and genotyping

C. elegans strains were cultured on bacterial lawns of OP50 and maintained at 12 to 20°C using standard methods (Brenner 1974) unless otherwise noted. The wild-type strain is N2 Bristol. The dat-1(ok157) III strain was a gift of J. Duerr and J. Rand (Oklahoma Medical Research Foundation, Oklahoma City) and is a complete loss-of-function mutation that eliminates the majority of the DAT-1 coding sequence. BY200 and BY250 are stable integrants (Nass et al. 2002) that express the transcriptional fusion Pdat-1::GFP (pRB490) on an N2 background. BY250 (vt67) was used for imaging DA neuron morphology of cloned and outcrossed swip lines and for 6-hydroxydopamine (6-OHDA) experiments. A line producing a loss-of-function disruption of DOP-3 (dop-3(vs106)x) was obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis) and cat-2(tm2261) from Shohei Mitani at the National Bioresource Project at the Tokyo Women’s Medical University. C. elegans genomic DNA was isolated as described previously (Nass et al. 2005) and used at a concentration of 1 ng/µL to genotype lines by polymerase chain reaction (PCR). For all crosses, either males were generated using the method originally described by Sulston and Hodgkin (Hodgkin 2005), or alleles were crossed to males of publically available strains containing integrated fluorescent transgenes that mark distinct nematode structures. After 24 hr of mating, hermaphrodites were separated onto individual plates and considered successful if ~50% of the progeny from an individual hermaphrodite are males and/or if the F1 progeny contained the fluorescent transgene from the male parent. For generation of the dat-1(ok157);vt29 strain, markers of LGIII and LGX were used, and the double-mutant genotype was confirmed by resegmentation of the two genes and confirmation with PCR genotyping of ok157 and SWIP testing for vt29. Generation of vt25,dat-1(ok157) will require recombination of the two alleles after identification of the vt25 functional variant and will be presented in a later report.

C. elegans assay for Swip

In both batch and automated analyses, we generated synchronous populations of these strains by hypochlorite treatment and harvesting arrested L1 animals. Early- to mid-stage L4 animals were identified by characteristic morphology and used for behavior because N2 animals show some stochastic Swip and quiescence bouts during the last larval molt. For automated analyses, single L4 hermaphrodites were placed in 20 µL of water in a single well of a Pyrex Spot Plate (Fisher; cat. no. 13-748B), and 10-min movies (uncompressed AVI format) of their swimming behavior were created and analyzed as described previously (Matthies et al. 2006; McDonald et al. 2007), with slight modifications.

We processed thrashing data using an in-house movement processing program (Worm Tracker, available on request) that fits a five-point spine to the worm in each frame. The five-point spines consist of four segments and three “joints.” In Worm Tracker, a spine is specified by the x and y positions of the spine center, the rotation of the spine with respect to the vertical axis, and finally the angles of the three joints. The spine positions are fit using a particle filter and motion detection. The Worm Tracker software processes a video and records the position of the fitted spine for each frame. A MATLAB script reads the exported files and computes the frequency of swimming using fast Fourier transform and by counting frames between angular extrema. This script then produces a data file that provides the frequency of motion of a given worm over time. These individual files are then grouped by genotype into a large data matrix and an accompanying annotation file made for each of the animals to be analyzed. These two files are then used as the input for a custom script written in the free publically available statistical program R. The program (SwimR, available upon request) smoothens individual data traces using a specified moving window and identifies any outliers in the sample file using a modified z-score calculation (Iglewicz and Hoaglin 1993). SwimR then generates several output text and PDF files, including a scatterplot of average frequency vs. time, a heat map of the samples ordered by strength of paralysis, and a histogram of the binned frequency data. For paralyzers, the script calculates the latency to paralyze and several parameters that define the ability of individual animals to revert from paralysis to regular thrashing activity, including
reversion probability (total time in reversion/total time after paralysis), time to first reversion, average reversion duration, and reversion strength (area under the curve during all reversion events). Parameters related to paralysis can be set by the user.

We defined paralyzed animals in our studies as those animals that fall below 20% of their maximal thrashing value and stay below this threshold for at least 20 sec. Revertants are defined as those animals that, after paralyzing, recover a threshold equivalent to 50% of their maximum thrashing rate for any length of time. For batch analysis, 10 to 20 worms were visually scored in a single well of the Pyrex Spot Plate. Worms displaying Swip after a 10-min assay period were counted and in some cases isolated by hand for further tests. For reserpine treatments, worms from each line were first synchronized by hypochlorite treatment, and L1 larvae are grown on OP50 plates containing 0.6 mM reserpine. After ~48 hr of reserpine or vehicle treatment, the population Swip analysis was repeated on groups of L4 animals from each line.

**Exogenous DA sensitivity assay**

Assays were performed as described in Chase et al. 2004, except that L4 animals were used in lieu of young adults because this stage is most relevant to the DAT-1–dependent Swip phenotype. To summarize, 10 L4 animals were transferred to 1% agar plates containing 2 mM glacial acetic acid and various concentrations of DA, incubated for 20 min, and then scored as paralyzed or moving. Animals were defined as moving if they were able to propagate a body bend through a minimum or maximum amplitude. All concentrations of DA were used on the same day.

**C. elegans mutagenesis screen**

Standard methods for a nonclonal, F2 screen were performed as originally described (Brenner 1974) and were used on wild-type hermaphrodite worms carrying a DAT-1 promoter–driven green fluorescent protein (GFP) transgene (BY200) (Nass et al. 2002). A semisynchronous population of healthy, well-fed late L4 animals was exposed to either 47 mM ethylmethanesulfonate (EMS) or 0.5 mM N-ethyl-N-nitrosourea at room temperature for 4 hr in a chemical fume hood. After 24 hr of recovery, 30 gravid adult worms were placed on each of eight 10-cm OP50 plates and allowed to lay ~50 eggs each (the F1s) before being discarded. After reaching adulthood and laying 20 to 30 eggs each for a total of 1000 to 1500 developing F2 animals per plate, the F1 animals were discarded. When F2 animals reached the L4 stage, they were batch screened for the Swip phenotype by rinsing off the plate and analyzing 50 to 100 animals per well as described previously. We tracked the source plate of each F2 so that only one stable mutant line was kept for each plate of the mutant F1s. After 10 min, animals that exhibited Swip were replated and allowed to recover. Swip-positive animals that recovered normal movement on solid media were cloned and tested in Swip assays to establish phenotype stability. Only lines in which at least 50% of the animals displayed Swip on retest were saved for a test of reserpine reversal of Swip, as described previously. In later rounds of screening, this convention was increased to 80% to improve recovery of stable lines. Stable lines that demonstrated a significant rescue of Swip after reserpine treatment were kept for further analysis. All recovered lines passing the reserpine test were outcrossed to the N2 strain a minimum of three times before further analysis. After each outcross, lines were rehomozygosed and retested for stable Swip on separate days with multiple parental founders before proceeding to the next cross. All lines recovered from the screen were sequenced with sense and antisense primers that span all DAT-1 exons and includes 1 kb upstream of the transcription start site as well as 50 bp downstream of the translational stop codon) using Big Dye Terminator Cycle Sequencing Mix (ABI, Foster City, CA). PCR products were sequenced on an ABI 3730xl DNA Analyzer (DNA Sequencing Core Facility, Vanderbilt Division of Medicine).

**SNP mapping**

Mapping of mutant loci was performed as described previously (Davis et al. 2005). In summary, stable outcrossed swip strains were crossed to the CB4856 strain. For bulk segregant analysis, lysates from both Swip-positive and Swip-negative F2 populations were generated and used as the input for genome-wide, 96-well PCR. N2 animals were not used as a control in these efforts because we found nonspecific N2 Bristol islands in both the Swip-positive and Swip-negative F2s on the left arm of LGI. The bulk segregant protocol was used to identify linkage groups that can serve for fine mapping with experiments replicated at least twice with separate populations to demonstrate consistent linkage. For fine-interval mapping, individual Swip-positive F2s were cloned, and their F3 progeny were tested for a stable Swip phenotype. Mutations were considered homozygous if the F3 population demonstrated Swip comparable to the original strain. Populations were manually scored in at least four to five assays using 40 to 50 worms. DNA from individual clones was then used as the input for PCR of individual intervals to ascertain a specific Bristol island on the mapped linkage group.

**Creation of plasmids and transgenic animals**

**Plasmids:** Pdat::GFP::DAT-1 (Carvelli et al. 2004) and Pdat::GFP (Nass et al. 2002) have been described previously. The following plasmids were created to examine DAT-1 mutations in heterologous mammalian expression systems: pRB1026 [DAT-1(G460D) in pcDNA3]; pRB1027 [DAT-1(W283*) in pcDNA3]; pRB1028 [N-terminally HA-tagged DAT-1(G460D) in pcDNA3]; and pRB1029 [N-terminally HA-tagged DAT-1(W283*) in pcDNA3] were developed to examine the DAT-1 mutations vta21 (G460D) and vta22 (W283*). pRB1030 [DAT-1(G460D) in pRB491] and pRB1031 [DAT-1(W283*) in pRB491] were created to evaluate the DAT-1 mutations vta21 (G460D) and vta22 (W283*) alleles in C. elegans in vivo. Lines shown in Figure 2 are ex21, ex62, and ex65 for DAT-1, vta21, and vta22. All mutations were created using the Quick Change XL site-directed mutagenesis kit (Stratagene, La Jolla, CA).

**Transgenic animals:** Fully sequenced constructs (90 ng/µL) were coinjected with the lin-15 rescuing plasmid pJM23 (40 ng/µL) into dat-1(OK157);lin-15(n765ts) animals using methods described previously (Jin et al. 1999). vtaIs18 is an outcrossed integrant of ex21 that expresses a Pdat::GFP::DAT-1, rescues the dat-1 strain, and maps to LGV.

**Mammalian cell culture and western blot analysis**

Methods for western blot analysis and surface biotinylation of the C. elegans DAT-1 expressed in COS-7 have been described elsewhere (Carvelli et al. 2004; Nass et al. 2005). In summary, COS-7 cells were plated and allowed to attach for 24 hr before transfection. Cells were transfected with 250 ng of either HA-tagged DAT-1 cDNA (pRB606) (Jayanthi et al. 1998), HA-tagged DAT-1(G460D) cDNA (vta21, pRB1028), HA-tagged DAT-1(W283*) cDNA (vta22, pRB1029), or an empty vector (pcDNA3; Invitrogen, Carlsbad, CA) using TransIT-LT1 (Mirus, Madison, WI) as the transfection vehicle. At 48 hr
Paralysis, or Swip (McDonald previously (Nass et al. 2007)). In brief, worms were mutagenized with EMS, and their F2 progeny batch were tested for Swip. Animals exhibiting Swip were individually cloned, and their progeny (F3s) were tested to determine Swip penetrance, scored as the percent of animals of a clonal population exhibiting Swip. Animals that failed to move normally on plates, and thus where Swip might reflect mutation of genes required for movement more generally (e.g., unc mutations), were eliminated. As a tertiary screen, we tested animals for Swip reversal after incubation with reserpine (McDonald et al. 2007). Because Swip in the dat-1 strain is fully penetrant, lines that exhibited ≥80% paralysis were pretreated with 0.6 mM reserpine, and animals were restested for Swip. From this tertiary analysis, we identified mutants that were either unaffected, rescued or, surprisingly, enhanced by reserpine pretreatment. To date we have sampled ∼10,000 mutant haploid genomes, and have isolated 25 primary mutant lines (vt20-vt44) that demonstrate a stable Swip phenotype and can be rescued by reserpine pretreatment. Of these 25, we found that 12 lines maintain their phenotype after 3X outcrossing, of which 10 lines are consistently rescued by reserpine pretreatment (Figure 3A). Here we report our genetic, biochemical, and behavioral analysis on four of these lines: vt21, vt22, vt25, and vt29.

To demonstrate that vt21, vt22, vt25, and vt29 possess normal DA neuron morphology, we examined their CEP, ADE and PDE neurons via expression of a transcriptional reporter (supporting information, Figure S1) and found that cell bodies and processes appear unaltered.

# RESULTS

## Forward genetic screen for reserpine-sensitive Swip phenotype

An artificial increase in extrasynaptic DA produced by the incubation of nematodes with exogenous DA leads to increased activation of inhibitory DA receptors expressed on cholinergic motor neurons (Chase et al. 2004), decreasing the release of acetylcholine from these neurons (Allen et al. 2011), resulting in paralysis. Our previous studies demonstrated that *dat-1(ok157)* worms display a paralytic phenotype in water in the absence of exogenous DA that we named Swimming Induced Paralysis, or Swip (McDonald et al. 2007). The Swip phenotype exhibited by *dat-1* animals is absent in *cat-2(TH::dat-1)* double mutants and can be rescued by pretreatment of animals with the *cat-1/VMA* inhibitor reserpine, a reagent that depletes vesicular DA stores (McDonald et al. 2007). Importantly, *dat-1* and *l dop-3* worms (Sugiura et al. 2005) also lack Swip behavior, establishing Swip as a phenotype that can derive from hyperdopaminergic signaling.

As schematized in Figure 1A, we hypothesized that incubation of worms in water (but not in isotonic medium; J. A. Hardaway and R. D. Blakely, personal communication) evokes DA release that in wild-type animals is limited in action by DAT-1. In *dat-1* animals, DA cannot be recaptured and spills over to extrasynaptic sites, where it can activate DOP-3 on motor neurons, decreasing cholinergic, neuromuscular signaling (Allen et al. 2011), producing Swip.

Because the Swip assay is a simple, rapid, and highly reproducible method for detecting genetic contributions to endogenous DA signaling, we implemented Swip in a forward genetic screen by using multiple, secondary tests to eliminate nonspecific mutants. As outlined in Figure 1B, L4 BY200 (Pdat-1::GFP) animals were mutagenized with EMS, and their F2 progeny batch were tested for Swip. Animals exhibiting Swip were individually cloned, and their progeny (F3s) were tested to determine Swip penetrance, scored as the percent of animals of a clonal population exhibiting Swip. Animals that failed to move normally on plates, and thus where Swip might reflect mutation of genes required for movement more generally (e.g., unc mutations), were eliminated. As a tertiary screen, we tested animals for Swip reversal after incubation with reserpine (McDonald et al. 2007). Because Swip in the *dat-1* strain is fully penetrant, lines that exhibited ≥80% paralysis were pretreated with 0.6 mM reserpine, and animals were restested for Swip. From this tertiary analysis, we identified mutants that were either unaffected, rescued or, surprisingly, enhanced by reserpine treatment. To date we have sampled ∼10,000 mutant haploid genomes, and have isolated 25 primary mutant lines (vt20-vt44) that demonstrate a stable Swip phenotype and can be rescued by reserpine pretreatment. Of these 25, we found that 12 lines maintain their phenotype after 3X outcrossing, of which 10 lines are consistently rescued by reserpine pretreatment (Figure 3A). Here we report our genetic, biochemical, and behavioral analysis on four of these lines: vt21, vt22, vt25, and vt29.
relative to N2 animals. These findings are consistent with our hypothesis that Swip is a hyperdopaminergic phenotype that is unlikely to derive from gross alterations in DA neuron circuitry.

Isolation of novel dat-1-null mutations

If our Swip screen is both specific and robust with respect to recovery of genes that control DA signaling, it should recover animals with dat-1 alleles, as well as novel mutants. To assess this issue, we first crossed vt21, vt22, vt25, and vt29 with dat-1(ok157), performing Swip complementation tests on their F1 progeny. From these experiments we found that N2 can complement dat-1 and each of the mutant strains (Figure S2A) and that vt25 and vt29 both complement dat-1 (Figure S2B) and one another (data not shown), indicating that vt25 and vt29 likely do not derive from mutations in dat-1. In contrast, vt21 and vt22 failed to fully complement Swip when crossed to dat-1(ok157) (Figure S2B). Likewise, when we crossed vt21 and vt22, we observed a failure to complement (data not shown), indicating that they are likely alleles of the same gene, that most likely is dat-1.

To test this hypothesis, we overexpressed GFP-tagged DAT-1(P\_dat-1::GFP:DAT-1) in vt21 and vt22 and tested for Swip in lines verified to express GFP in DA neurons. As previously demonstrated for the DAT-1 deletion allele dat-1(ok157) (McDonald et al. 2007), transgenic expression of GFP-DAT-1 rescued the Swip phenotypes of vt21 and vt22 (Figure 2A), suggesting that these mutants reduce DAT activity. Whereas sequencing of the genomic DAT-1 locus in vt25 and vt29 yielded a sequence identical to that found in N2 animals, vt21 and vt22 were found to bear single base-pair substitutions that were predicted to impact the DAT-1 coding sequence (Figure 2B).

The DAT-1 gene contains 13 exons that encode a 615 amino-acid protein with 12 transmembrane domains and intracellular N and C-termini (Jayanthi et al. 1998). vt21 bears a G→A substitution at
In vt21, a G→A transition at nucleotide 2395 results in the conversion of a highly conserved glycine (G) to aspartic acid (D). (C–E) Expression and trafficking deficits of vt21 and vt22 alleles of dat-1. Animals injected with Pdat-1::GFP:DAT-1 (C) display readily detectible expression and localization to CEP and ADE soma (white arrows) and terminals (white triangles) whereas mutant transporters (D and E) are much more weakly expressed and restricted in localization to DA neuron cell bodies. L4 animals were anesthetized and Z-stacks of CEP/ADE neurons were acquired to illustrate the conversion of tryptophan (T) to a premature stop codon (†).

To examine the deleterious impact of the DAT-1 mutations identified in vt21 and vt22 lines, we implemented a previously described heterologous expression protocol (Nass et al. 2005). Our assay of [3H]DA transport activity in COS-7 cells revealed that both dat-1(vt21) and dat-1(vt22) display significantly reduced DA uptake relative to that displayed by WT HA:DAT-1 (Figure S3C). Whereas dat-1(vt22) produced no significant [3H]DA uptake above nontransfected cells, dat-1(vt21) exhibited low, but detectible, DA transport activity (Figure S3C). Analysis of total and cell-surface protein extracts demonstrated that dat-1(vt21) and dat-1(vt22) exhibit reduced cell-surface expression levels and a premature truncation product respectively relative to WT DAT-1 (Figure S3D). Furthermore, whereas GFP:DAT-1 expression rescued the Swip phenotype of dat-1(ok157) (Figure S3E), neither GFP:DAT-1 (G460D) nor GFP:DAT-1(W283stop) rescued Swip. Confocal analysis of wild-type GFP:DAT-1 expression in CEP neurons revealed moderate levels of transporter expression in DA cell bodies (Figure 2C, white arrows), low levels in dendrites and axons, and high expression in presynaptic terminals (white arrowheads). In contrast, GFP:DAT-1(G460D) demonstrated robust CEP cell body expression but failed to label CEP processes and terminals (Figure 2D). Animals expressing GFP:DAT-1(W283stop) demonstrated lower levels of GFP signal than was observed in GFP:DAT-1 or GFP:DAT-1 (G460D), with all labeling confined to CEP cell bodies (Figure 2E).

Together, these findings provide in vitro and in vivo support for the determinants of Swip in the vt21 and vt22 lines as derived from dat-1 alleles. Because we did not observe mutations in DAT-1 in vt25 and vt29, we further mapped the sites of these mutations as described in Materials and Methods. We found that vt25 maps to LGIII, whereas vt29 maps to LGX (Figure S4). No genes known to impact DA signaling lie in the region mapped in vt25. The vt29 region harbors the dop-4 gene. Sequencing of this gene in vt29 animals revealed no mutations, indicating that the altered DA signaling of vt29 is produced through a novel mechanism. These findings confirm that vt25 and vt29 do not harbor Swip-causing dat-1 alleles, nor do they involve mutations in the same gene.

vt25 and vt29 exhibit dat-1-like locomotive behaviors but distinct sensitivities to exogenous DA

To determine the similarity of vt25 and vt29 behavior to that of dat-1, we conducted a series of DA signaling–dependent locomotor tests. We also assayed dat-1(vt21) and dat-1(vt22) in parallel to explore whether this test might reveal dat-1 allele-specific effects. Using an automated assay of swimming behavior (Matthies et al. 2006; McDonald et al. 2007), we found that vt25 lacked a fully penetrant Swip phenotype, as evident by its ∼50% paralysis in manual scoring (Figure 3A) and an average ∼0.5 Hz thrashing frequency by our final assay time point (10 min) under automated recording (Figure 3C). In contrast, vt29 displayed fully penetrant Swip, yielding ∼100% paralysis in manual scoring assays (Figure 3A), and an even more rapid rate of paralysis than dat-1 (Figure 3C and Table 1). In these
Figure 3: vt25 and vt29 demonstrate alterations in DA-dependent locomotory behaviors. (A) After a screen of ~10,000 haploid genomes, 25 vt mutant lines were outcrossed, of which 10 were found to maintain their reserpine sensitivity. Populations of at least 10 worms were assayed in a single well and after 10 min, with animals scored as no. animals swimming/total animals. Each bar represents at least 20 assays performed over several experimental days by multiple, blinded experimenters. Drug treatment effects were analyzed using an unpaired Student’s t-test comparing against basal Swip activity for that line, where *P < 0.05, **P < 0.01, and ***P < 0.001 and error bars represent SEM. (B) Dat-1 and vt29 displayed enhanced sensitivity to exogenous DA on a solid substrate, whereas vt25 maintained an N2-like DA dose response. For these assays, 10 L4 stage worms were placed on plates containing increasing concentrations of exogenous DA, incubated for 20 min, and then scored for 10 sec as paralyzed or moving. Dose–response curves were compared using two-way ANOVA with Bonferroni posttests comparing mutants to N2, where dat-1 + vt29 were all found to be significantly different from N2 with a P < 0.001 at 15 + 20 mM DA. Data derive from at least eight tests per strain per concentration. Error bars represent SEM. (C) vt25 demonstrates a greater average frequency of swimming at later time points as compared with dat-1 animals, whereas vt29 paralyzes faster. Individual animals were recorded using a video capture system and then analyzed with custom-designed Thrasher software that assigns multiple linear elements projecting from the worm centroid. The position of these linear elements are tracked and converted off-line to movement frequency as a function of time. Batch conversions are generated, providing mean values and SEM along moving averages. Error bars are not shown in these plots for simplicity. Average thrashing plots were analyzed using two-way ANOVA with Bonferroni’s multiple comparison analysis. vt25 and vt29 swimming frequencies were found to be significantly reduced from N2 after 58 sec and 39 sec, respectively. Although we observed no overall significant difference in these assays in comparing dat-1(ok157) and vt25, the thrashing frequency of vt29 was found to be significantly reduced from dat-1(ok157) between 52 sec and 2 min 25 sec. (D) Swip behavior of vt25 and vt29 is dependent on DOP-3 and CAT-2, respectively. The figure represents the mean swimming behavior as measured by manual scoring. Data derive from observations from >24 trials for each strain. For vt25; dop-3(vs106) and cat-2(tm2261); vt29, the vt genotypes were confirmed via an independent complementation test. Data were analyzed using one-way ANOVA with multiple Bonferroni posttests where ***P < 0.001 and error bars represent SEM.
and further assays, we determined that \textit{dat-1(vt21)} and \textit{dat-1(vt22)} behave similar to the \textit{dat-1(ok157)} strain (Figure S5 and Table 1).

The Swip of \textit{dat-1} animals requires the function of the \textit{Go_\alpha_2} coupled DA receptor \textit{DOP-3} (Chase et al. 2004; McDonald et al. 2007; Allen et al. 2011) that is expressed by body wall muscle and ventral cord motor neurons. As with \textit{dat-1} alleles, \textit{dat-1(vt21)} and \textit{dat-1(vt22)} paralysis was completely suppressed in a cross to \textit{dop-3} (Figure S5). \textit{Vt25} paralysis was modestly but significantly rescued by \textit{dop-3} (Figure 3D). We could not examine rescue of \textit{vt29} with \textit{dop-3} because they both map to the same chromosome. Therefore, we crossed this line to a line deficient in the rate-limiting enzyme in DA biosynthesis, tyrosine hydroxylase (\textit{cat-2}), as \textit{cat-2} suppresses the Swip of \textit{dat-1(ok157)} (McDonald et al. 2007). As with \textit{dat-1} alleles, we found that \textit{cat-2} completely suppressed the paralysis behavior of \textit{vt29} (Figure 3D).

The application of exogenous DA to worms on solid substrate induces motor slowing and paralysis (Schafer and Kenyon 1995). We found that \textit{dat-1} animals transferred to plates containing increasing amounts of DA displayed a twofold increase in DA sensitivity when compared with \textit{N2} animals (Figure 3B and Figure S5), possibly reflecting a lack of \textit{DAT-1}-dependent clearance of exogenous DA once the catecholamine permeates the cuticle. \textit{Vt29} displayed sensitivity to exogenous DA like that of \textit{dat-1} (Figure 3B). In contrast, \textit{vt25} exhibited a DA dose-response more similar to \textit{N2} (Figure 3B).

### Automated analysis of Swip behavior reveals a differential impact on thrashing behavior for \textit{vt25} and \textit{vt29}

To more precisely quantify the thrashing behavior of our \textit{vt} mutants, we developed software tools (SwimR; see Materials and Methods, J. A. Hardaway and J. Wang, unpublished data) that can provide for a more detailed kinetic analysis of individual animals that may reveal patterns of behavior not readily detected in population averages (Table 1). For visualization, our software plots each animal’s swimming behavior horizontally (Figure 4A), assigning a color code that ranges from red (high frequency) to green (low frequency) for each time block and that orders the animals within each genotype so that more rapid paralyzers are displayed at the bottom and slower paralyzers (or relatively constant swimmers) are displayed at the top. These analyses revealed several significant differences between the mutants. All mutants recovered in the screen demonstrated significantly reduced maximal thrashing frequencies in water, with \textit{vt29} being the most impacted, and accompanied by a greater percentage of animals displaying lower thrashing frequency values (Figure S6). With respect to paralysis, \textit{vt25} animals again resembled \textit{dat-1}, whereas \textit{vt29} differed from these lines with a significantly reduced latency to paralyze. Although by definition, all lines paralyze in water, our single-worm analyses revealed that \textit{dat-1} and \textit{vt25} lines feature a significant number of animals that revert back to relatively normal swimming behavior that we tabulated as reversion incidence (no. revertants/no. paralyzed) and reversion probability (time in reversion/time after paralysis onset).

Table 1 Kinetic attributes of paralysis and reversion

|          | Maximal Frequency, Hz | Latency to Paralyze, sec | Reversion Incidencea | Reversion Frequency, Events/Animal | Reversion Probabilitya | Time to First Reversion, sec | Average Reversion Event Length, sec |
|----------|----------------------|--------------------------|----------------------|-----------------------------------|------------------------|-------------------------------|----------------------------------|
| \textit{N2} (n = 52) | 1.76 ± 0.01 | N/A | N/A | N/A | N/A | 145 ± 35.8 | 5.77 ± 1.4 |
| \textit{dat-1(ok157)} (48) | 1.45 ± 0.04* | 174 ± 17.5 | 0.25 | 3.22 ± 0.571 | 0.031 ± 0.006 | 145 ± 35.8 | 5.77 ± 1.4 |
| \textit{vt25} (47) | 1.28 ± 0.04*** | 131 ± 14.5 | 0.391 | 2.00 ± 0.271 | 0.020 ± 0.004 | 112 ± 17.7 | 4.54 ± 0.93 |
| \textit{vt29} (37) | 1.13 ± 0.04*** | 81.2 ± 6.05** | 0.0540d* | 2.50 ± 0.500 | 0.018 ± 0.007 | 146 ± 108 | 3.59 ± 0.69 |
| \textit{vt18} (42) | 1.64 ± 0.02ns | N/A | N/A | N/A | N/A | N/A | N/A |
| \textit{vt25}; \textit{vt18} (38) | 1.45 ± 0.03 | 337 ± 61* | 0.333 | N/A | N/A | N/A | N/A |
| \textit{vt29}; \textit{vt18} (44) | 1.45 ± 0.02 | 165 ± 22.4 | 0.294 | 3.90 ± 0.706 | 0.051 ± 0.019 | 107 ± 30.7 | 4.43 ± 1.14 |
| \textit{M9} | 1.76 ± 0.01 | N/A | N/A | N/A | N/A | N/A | N/A |
| \textit{dat-1(ok157)} (56) | 1.66 ± 0.02 | 166 ± 34.1 | 0.810d ### | 5.06 ± 0.774 | 0.052 ± 0.014 | 110 ± 11.7 | 4.18 ± 1.10 |
| \textit{vt29} (53) | 1.29 ± 0.03*** | 298 ± 24.5 | 0.640d ### | 4.19 ± 0.647 | 0.040 ± 0.008 | 171 ± 25.7 | 4.20 ± 0.76 |

Asterisks indicate a significant difference to \textit{dat-1(ok157)}, as determined by one-way analysis of variance with Bonferroni’s multiple comparison test, where **P < 0.05, ***P < 0.01, and ****P < 0.001. Numerals signs (#) indicate a comparison within the same genotype between water and \textit{M9}.

\(a\) Reversioni = fraction of animals that reverse from paralysis.

\(b\) Reversion probability = total time spent in reversion/total time after paralysis onset.

\(c\) Comparison with \textit{N2} (ns = not significant).

\(d\) \(x^2\) test.

\# Evaluation of \textit{vt25} and \textit{vt29} sensitivity to osmolarity

Previously, we demonstrated that the \textit{dat-1} Swip phenotype is highly dependent on the osmolarity of the swimming medium (J. A. Hardaway, personal communication to Worm Breeder’s Gazette), with a near-complete loss of Swip when these animals are subjected to aqueous solutions buffered to 300 mOsm with sucrose, or in \textit{M9} (325 mOsm). We used manual scoring of the fraction of animals paralyzed at 10 min to assay swimming behavior of \textit{N2}, \textit{dat-1}, \textit{vt25}, and \textit{vt29} as a function of medium osmolarity. \textit{N2} animals, as expected, maintained a relatively constant swimming rate regardless of osmolarity (Figure 5A), whereas \textit{dat-1} animals displayed the expected loss of paralysis with increasing osmolarity. \textit{Vt29} animals exhibited a virtually identical sensitivity to osmolarity as \textit{dat-1}, except a slight, yet significantly increased paralysis at greater osmolarities. Remarkably, \textit{vt25} animals displayed a lack of sensitivity to the osmolarity of solutions used in swimming assays. Although only minimal
paralysis of \textit{dat-1} and \textit{vt29} animals was detected in M9 medium using manual scoring, automated analyses revealed that both \textit{dat-1} and \textit{vt29} exhibited reduced average rates of movement (Figure 5B). At the single-worm level, several parameters distinguished \textit{dat-1} and \textit{vt29} in M9 medium (Figure 5, C–F; Table 1). \textit{Vt29} animals could not achieve the same maximal swimming frequency as \textit{dat-1} animals in M9, and unlike water, \textit{dat-1} maximal rates were not significantly different from N2 (Table 1). In M9, \textit{vt29} animals exhibited a significantly greater latency to paralyze than \textit{dat-1} animals (Table 1 and Figure 5D), opposite to their relative sensitivities in water. Both \textit{dat-1} and \textit{vt29} lines demonstrated an increase in the number of revertants in M9 as compared with water (Figure 5E) without changing the length of reversion events (Figure 5F), although this effect was greatest in \textit{vt29} animals. Therefore, \textit{vt29} are more likely to paralyze in M9 than \textit{dat-1} but as a population reach paralysis more slowly and are more likely to reverse from paralysis than when assayed in water.

**Suppression of Swip in \textit{vt25} and \textit{vt29} with DAT overexpression**

Although we did not detect mutations at the DAT-1 locus in \textit{vt25} and \textit{vt29}, these mutations may still impact DA clearance, either through a functional impact on DAT-1 at DA terminals or through changes in the somatic export of DAT-1 protein. To assess this issue, we crossed \textit{vt25} and \textit{vt29} onto a line containing an integrated \textit{p\textit{dat-1}::GFP:DAT-1} transgene and monitored swimming behavior. Overexpression of GFP:DAT-1 restored swimming behavior of \textit{vt25} (Figure 6A) to near wild-type levels. Single-worm analyses (Figure 6, B and C) demonstrated that although Swip is largely rescued when overexpressing DAT on the \textit{vt25} background, short paralytic bouts are still evident. GFP:DAT-1 overexpression only modestly suppressed \textit{vt29}. Automated analyses revealed that the partial suppression of \textit{vt29} by overexpression of GFP:DAT-1 does not derive from an increased reversion probability or latency to paralyze (Table 1) but rather appears to result from a reduction in Swip penetrance, with a reduced fraction of animals swimming at or near the rates observed with \textit{vt29} alone (Figure 4A compared with Figure 6C). To examine whether the findings from the rescue experiments with \textit{p\textit{dat-1}::GFP:DAT-1} reflect a failure to synthesize or traffic the transporter, we examined the impact of \textit{vt25} and \textit{vt29} backgrounds on the localization of GFP:DAT-1 to the soma, dendrites, axons, and presynaptic terminals of CEP, ADE, and PDE neurons. We could detect no changes in the pattern of GFP:DAT-1 localization with the \textit{vt25} and \textit{vt29} strains, comparing animals with GFP:DAT-1 expressed on an otherwise wild-type background to \textit{vt25}, \textit{vt29}, and \textit{dat-1} have reduced tissue DA content

One reason for \textit{vt29} to possess a stronger, DA-dependent Swip phenotype than \textit{dat-1(ok157)} would be for \textit{vt29} to store excess DA in synaptic vesicles that, upon release, could produce greater levels of extrasynaptic DA than seen with wild-type DA levels and a loss of DAT-1-mediated DA clearance. To investigate this issue, we measured DA levels in \textit{vt25} and \textit{vt29} animals in parallel with assays of N2, \textit{dat-1(ok157)}, and \textit{cat-2(tm2261)} lines. As expected, \textit{cat-2(tm2261)}
displayed a highly significant reduction in DA levels compared with N2 (Figure 6D). Dat-1 DA levels were also reduced, in keeping with findings of reduced tissue DA content in DAT knockout mice believed to derive from a need to recycle extracellular DA to maintain vesicular stores (Giros et al. 1996). Interestingly, both vt25 and vt29 also displayed reduced DA levels, suggesting that, analogous to dat-1, vt25, and vt29 animals produce a hyperdopaminergic phenotype that precludes the maintenance of normal presynaptic DA levels.
vt25 and vt29 demonstrate differential protection against 6-OHDA neurotoxicity in vivo

The reduction in DA levels in vt25 and vt29, in the context of a hyperdopaminergic phenotype, raised the possibility that the mutations harbored by these lines produces a loss of DAT-1 loss function that might be masked by other effects and thus not detected via the methods we had used to this point. Our laboratory previously demonstrated that treatment of worms with 6-OHDA results in a necrotic loss of DA neurons and that this process requires transport of the compound by presynaptic DAT-1 to produce neural degeneration (Nass et al. 2002). This phenotype presented us with an opportunity to more directly examine the function of DAT-1 in vt25 and vt29 lines in vivo. Consistent with our published studies, we observed degeneration of DA neurons in N2, but not dat-1 animals, after an acute (1 hr) treatment with 6-OHDA (Figure 6E). 6-OHDA was significantly less effective in lesioning DA neurons in vt25 animals
compared with N2, suggesting that the gene responsible for Swip in this line normally plays a role in modulating DAT-1 activity. In contrast, vt29 demonstrated full sensitivity to the toxin, consistent with the mutation in this line impacting a process parallel to DAT-1 in constraining DA signaling.

**Dat-1 and vt29 exhibit additivity for Swip when assayed under hypotonic conditions**

With evidence that vt29 impacts DA signaling in a DAT-1-independent pathway, we returned to our assessment of Swip behavior under hypotonic conditions, examining paralysis at an intermediate osmolarity using single and double mutant lines. At a medium osmolarity of 100 mOsm, we observed a significant increase in Swip in the *dat-1;vt29* double mutant, as compared with the Swip penetrance of *dat-1* or vt29 alone (Figure 6F) and consistent with the mutation harbored by vt29 impacting a pathway parallel to that impacted by *dat-1* (Figure 6G).

**DISCUSSION**

The *C. elegans* gene T23G5.5 (DAT-1) was the first invertebrate SLC6 family member to be cloned and characterized, revealing structural and functional characteristics similar to those of vertebrate catecholamine transporters (Jayanthi et al. 1998). *In vitro* heterologous expression studies revealed DAT-1 to exhibit substrate specificity for DA over other catecholamines and to be antagonized by imipramine, amphetamine, and cocaine. Subsequent *in vivo* efforts (Nass et al. 2002) demonstrated that a 700-bp DNA element immediately upstream of the DAT-1 transcription start site confers expression of GFP in all *C. elegans* DA neurons (CEP, ADE, PDE in the hermaphrodite). Nass and coworkers capitalized on the visibility of DA neurons in living nematodes to monitor neurotoxin (6-OHDA)-induced DA neuron degeneration and demonstrate the dependence of cell death on toxin accumulation by DAT-1.

The discovery of a DAT-1-dependent phenotype provided an initial opportunity to implement forward genetic approaches for the identification of 6-OHDA toxicity suppressors (Nass and Blakely 2003; Nass et al. 2005). As the basis for this screen derives from sensitivity to an exogenous agent, the utility of the 6-OHDA screen for the elucidation of molecules regulating endogenous, DAT-1-dependent DA synaptic events is limited. Therefore, we sought to identify behavioral alterations arising from compromised, endogenous DA signaling, as revealed in studies of a genetic loss of DAT-1. Although loss of DAT in mice produces a dramatic motor phenotype (Giros et al. 1996), and exogenous DA can paralyze worms (Schafer and Kenyon 1995; Savin et al. 2000; Chase et al. 2004), deletion of a large portion of the *C. elegans* DAT-1 gene fails to induce an overt motor phenotype on solid substrate. DA neurons in the worm are mechanosensitive (Savin et al. 2000), and thus we reasoned that insufficient physical stimulation is present under normal culture conditions to elicit sufficient DA release, thereby reducing the sensitivity of measures focused on a *dat-1* mutation. Wild-type worms placed in water thrash rapidly for 20 min or longer, and we considered whether this more vigorous mode of motion might trigger more elevated DA release. Indeed, when we placed *dat-1* (ok157) animals in water, we discovered that these animals swim briefly at normal rates and then paralyze—a phenotype we termed Swip (McDonald et al. 2007). Swip in *dat-1* animals requires the biosynthesis of endogenous DA, packaging of DA into synaptic vesicles, and activation of the DOP-3/Gesa pathway (McDonald et al. 2007). The current study confirms that Swip can be used to screen for molecular alterations that impact DAT-dependent clearance of DA.

In our initial screen, many of the lines we identified, when retested in a subsequent generation, demonstrated incomplete penetrance of the Swip phenotype, and, thus we chose to save only those clones in which at least 80% of animals in subsequent generations exhibited Swip. The current report represents an analysis of >10,000 haploid genomes, where we uncovered 10 stable lines originating from mutations in at least three distinct genes, as determined through direct sequencing, rescue experiments, mapping, and complementation analyses. Visualization of GFP-labeled DA neurons on the mutant backgrounds verified that gross morphologic deficits of DA neurons do not account for the Swip phenotype of the lines presented in the current report. Our design also reduced the probability that mutations recovered would arise in shared elements of neurotransmitter release machinery or determinants of cholinergic and GABAergic signaling, as these lines would be predicted to exhibit locomotor dysfunction on solid substrate. Evidence that the lines recovered in our screen (1) display *cat-2* and/or *dop-3* reversal; (2) can be rescued by the presynaptically directed vMAT/cat-1 inhibitor reserpine; (3) include two novel *dat-1* alleles; (4) produce changes in DA levels (for vt25 and vt29); and (5) (for vt25) reduce 6-OHDA sensitivity lead us to infer that the approach we have used provides for a preferential identification of presynaptically acting genes. Of course, we cannot rule out that our design and subsequent tests could recover mutants acting postsynaptically, although it seems more likely that a screen to identify suppressors of *dat-1*-mediated Swip would be more useful in recovering such animals (Wani et al. 2012). Finally, although we chose not pursue them, we also identified multiple lines that exhibited enhanced Swip after reserpine treatment, which could reflect an influence of octopamine, tyramine, or serotonin, as these neurotransmitters can also modulate motor activity and their vesicular packaging by CAT-1 should also be reserpine-sensitive (Duerr et al. 1999; Alkema et al. 2005).

Two of the lines recovered in our screen harbor point mutations in the coding region of DAT-1. Vt22 bear a nonsense mutation, predicted to truncate the DAT-1 protein at Trp283, whereas vt21 possesses a missense mutation predicted to generate a nonconservative substitution of Gly for Asp at amino acid 460. All eukaryotic SLC6 family members possess a Gly residue at this position, although ours is the first study to demonstrate a functional significance of this site *in vivo*. Importantly, our studies of vt21 and vt22 provide critical proof of concept that a Swip-based, forward genetic screen can target genes critical for the regulation of DA signaling.

In addition to the isolation of new *dat-1* alleles, we present a functional characterization of two additional stable lines exhibiting Swip, vt25 and vt29. These two lines share reserpine-sensitive Swip with *dat-1*(ok157), *dat-1*(vt21) and *dat-1*(vt22), and like these mutants, their Swip is suppressed by *dop-3* or *cat-2* mutations. Complementation analyses and mapping of the mutant genes harbored by vt25 and vt29 revealed that they bear mutations in two independent loci that do not overlap or contain mutations in known regulators of DA signaling and thus likely represent novel genetic components of DA signaling.

In parallel with *dat-1*(vt21) and *dat-1*(vt22), we assayed the response of vt25 and vt29 to increasing doses of exogenous DA. Relative to N2, all *dat-1* mutants, as well as vt29, exhibited a statistically significant, twofold increase in sensitivity to exogenous DA on solid substrate. In contrast, vt25 demonstrated a more N2-like response to DA, our first indication that these genes support DA signaling via separate pathways. The molecular mechanisms supporting a hyper-sensitivity to exogenous DA are yet to be determined, though in the case of the *dat-1* mutants, a plausible basis is a reduced requirement for exogenous DA to achieve DOP-3 activation in animals that cannot
capitalize on the DA clearance activity provided by DAT-1. This mechanism has some support from the demonstration that DAT-1 can accumulate other exogenous substrates, such as the dopaminergic toxin 6-OHDA (Nass et al. 2005). A second possibility is that, in DAT-1−deficient animals, chronically elevated levels of extrasynaptic DA may lead to a desensitization of a DA autoreceptor that may normally act to suppress DA release via a negative-feedback mechanism. In mammals, the D2 receptor serves both to suppress DA neuron firing and to reduce DA release (Usiello et al. 2000; Schmitz et al. 2002; Beckstead et al. 2004; Bello et al. 2011). Although such a mechanism has not been defined in nematodes, a D2-like DA receptor DOP-2 is also expressed by C. elegans DA neurons (Suo et al. 2003). By inference, desensitization of presynaptic DOP-2 produced by constitutively elevated extracellular DA could result in enhanced DA release that synergizes with exogenous DA, thereby reducing the amount of DA needed on plates needed to produce immobility. Studies are needed in which investigators use lines with DA neuron-specific elimination of DOP-2 to test this possibility. Such animals would also be useful in testing whether mutants recovered in our screen produce their hyperdopaminergic phenotype by suppressing DOP-2 mediated, inhibitory control of DA neuron excitability and/or DA release.

As our studies progressed, we recognized that our automated movement analysis software (Tracker) could be augmented to provide a much more detailed analysis of activity patterns than is reported in standard thrashing assays. To accomplish this goal, we developed a new suite of analytical tools that would allow for an unbiased assessment of the behavior of animals, both as individuals and across a population. Although the essential patterns among the strains we assayed were consistent, finer aspects of their swimming behavior were now observable. One such novel observation concerns the finding that a significant fraction of dat-1 animals spontaneously recovered from paralysis for short intervals (termed here "reversals"). Because we observe these reversals in the context of a hyperdopaminergic phenotype, it is possible that excess postsynaptic DA signaling produces reversals in the course of moving between two distinct behavioral states. In this regard, Vidal-Gadea et al. 2011 demonstrated that DA is necessary and sufficient for C. elegans transit from swimming to crawling.

Whereas the Swip of dat-1 animals is dependent solely on DOP-3, the swim-crawl transition requires DOP-1 and DOP-4 (Vidal-Gadea et al. 2011). Alternatively, reversals may derive from recruitment of other circuits that stimulate movement and that become evident when other circuits that stimulate movement and that become evident when

The kinetic differences in Swip behavior observed for vt25 and vt29 raised the question as to whether the strength of Swip in these lines is maintained under conditions in which Swip is suppressed in the dat-1 strain. Previous study of the environmental dependence of the dat-1 Swip phenotype revealed that an increase in the osmolality of the swimming media significantly suppresses the Swip phenotype (I. A. Hardaway, personal communication to Worm Breeder’s Gazette). In this study, we were able to replicate that finding by using both manual assays and automated thrashing analysis. Remarkably, vt29 maintained its Swip behavior across all osmolarities, whereas vt29 swimming behavior, like dat-1, was recovered as the osmolality of the medium approached that of M9. We also assayed the behavior of the dat-1; vt29 double mutant across these osmolarities and observed an increase in Swip penetration at intermediate osmolarities.

These findings suggest that dat-1 and vt29 act in parallel, independent pathways that can sum to produce an additive paralytic response at intermediate osmolarities. Although both the dat-1 and vt29 strain demonstrated recovery of swimming in the isotonic buffer M9, our automated analysis demonstrated that, nonetheless, dat-1 behavior could be differentiated from vt29 in that the latter line maintains a stronger Swip phenotype even in media of high osmolality. It is commonplace to use salt-buffered solutions in the study of neurotransmitter regulation of swimming, despite the fact that the organism normally resides in an environment with variable ionic content. Together, these studies demonstrate that the attribution of genes to DA signaling pathways, as well as their epistasis, will benefit from the use of osmotic profiling.

To investigate the potential pathways in which vt25 and vt29 reside, we assayed their Swip phenotypes in the context of DAT-1 transgenic overexpression. This transgene fully rescues the dat-1 mutant (McDonald et al. 2007) and almost completely restored the swimming behavior of vt25, although short transient bouts were still evident. In contrast, vt29 swimming behavior was only partially restored by DAT-1 overexpression. Because the vt25 strain displays a weaker phenotype, the relative magnitude of suppression between vt25 and vt29 is roughly equivalent and suggests that overexpression of DAT can partially restore disruptions in multiple pathways regulating DA homeostasis. Manipulation of multiple DAT-interacting proteins have been implicated in the folding, trafficking, stability, and activity of the transporter, including PICK1 (Torres et al. 2001), Hic-5 (Carneiro et al. 2002), syntaxin1A (Lee et al. 2004), RACK1 (Lee et al. 2004), α-synuclein (Lee et al. 2001), synaptogyrin-3 (Egaña et al. 2009), and the D2 DA receptor (Lee et al. 2007), although the significance of these interactions in vivo has not been studied. Mutation of the C. elegans orthologs (where they exist) of these genes is unlikely to contribute to the phenotypes of vt25 and vt29 based on their absence from mapped loci. In addition, because we observed no difference in the somatic export of GFP-DAT-1, we doubt they produce Swip through alterations in transporter trafficking to the synapse. This is particularly evident with vt29 animals that retained the full 6-OHDA sensitivity exhibited by N2. However, vt25 displayed
a more moderate 6-OHDA sensitivity and thus part of its actions may involve local, synaptic DAT-1 trafficking to the plasma membrane or control of transporter activity.

Finally, as vt25 or vt29 display a hyperdopaminergic signaling phenotype, release of enhanced, synthesized, or stored DA could explain their phenotypes. However, when we measured the DA content of the molecular lesions borne by normally exerting suppression of DA neuron excitability and/or DA f o r g e n a t i o n o f D A T - 1 s t r u c t u r a l m o d e l s . T h e a u t h o r s a l s o a c k n o w l -
and expert technical assistance, and Julie Field and Kristian Kaufmann that our efforts in this regard may identify molecules whose intensive
vation of components of DA signaling throughout phylogeny suggests that now can be targeted in future studies, is that the membrane or control of transporter activity.

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