# Coexpressed D1- and D2-Like Dopamine Receptors Antagonistically Modulate Acetylcholine Release in *Caenorhabditis elegans*

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**ABSTRACT** Dopamine acts through two classes of G protein-coupled receptor (D1-like and D2-like) to modulate neuron activity in the brain. While subtypes of D1- and D2-like receptors are coexpressed in many neurons of the mammalian brain, it is unclear how signaling by these coexpressed receptors interacts to modulate the activity of the neuron in which they are expressed. D1- and D2-like dopamine receptors are also coexpressed in the cholinergic ventral-cord motor neurons of *Caenorhabditis elegans*. To begin to understand how coexpressed dopamine receptors interact to modulate neuron activity, we performed a genetic screen in *C. elegans* and isolated mutants defective in dopamine response. These mutants were also defective in behaviors mediated by endogenous dopamine signaling, including basal slowing and swimming-induced paralysis. We used transgene rescue experiments to show that defects in these dopamine-specific behaviors were caused by abnormal signaling in the cholinergic motor neurons. To investigate the interaction between the D1- and D2-like receptors specifically in these cholinergic motor neurons, we measured the sensitivity of dopamine-signaling mutants and transgenic animals to the acetylcholinesterase inhibitor aldicarb. We found that D2 signaling inhibited acetylcholine release from the cholinergic motor neurons while D1 signaling stimulated release from these same cells. Thus, coexpressed D1- and D2-like dopamine receptors act antagonistically *in vivo* to modulate acetylcholine release from the cholinergic motor neurons of *C. elegans*.

DOPAMINE (DA) modulates neural activity in the mammalian brain by acting through two classes of G protein-coupled receptors, with D1 and D5 receptors in the D1-like class and D2, D3, and D4 receptors in the D2-like class. Pharmacological agents that distinguish between classes of receptor, but not between receptors within a class, have been used to show that signaling by D1- and D2-like receptors can have synergistic or antagonistic effects on gene expression and behavior (Plaznik *et al.* 1989; Keefe and Gerfen 1995; Kelly *et al.* 1998; Gong *et al.* 1999; McNamara *et al.* 2003). The cellular and molecular mechanisms that underlie these effects have not been clearly established and are likely to be difficult to dissect as many neurons in the brain express more than one DA receptor and the G proteins and signaling pathways activated by DA receptors vary depending on the region of the brain and type of neurons in which DA receptors are expressed (Stoof and Kebabian 1981; Undie and Friedman 1990; Surmeier *et al.* 1992; Jin *et al.* 2001). Understanding how signaling pathways regulated by coexpressed DA receptors interact to modulate neural function is critical to understanding how abnormal DA signaling in the brain contributes to neurological disorders including schizophrenia and Parkinson's disease.

In *Caenorhabditis elegans*, DA is made and released from eight mechanosensory neurons and acts extrasynaptically to control behavior (Sulston *et al.* 1975; Chase *et al.* 2004; Sanyal *et al.* 2004). DA acts through D1-like (DOP-1) and D2-like (DOP-3) DA receptors in *C. elegans*, and these receptors are expressed on distinct neurons but are also coexpressed on some neurons (Suo *et al.* 2002; Chase *et al.* 2004). Orthologs of each of the major G proteins that couple to DA receptors in mammals, including Gαs, Gαi/o, and Gαq, are expressed throughout the *C. elegans* nervous system (Jansen *et al.* 1999).

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*C. elegans* movement is modulated by DA acting through DOP-1 and DOP-3 receptors such that signaling through DOP-3 and Gαo inhibits locomotion, and signaling through DOP-1, Gαq, and PLCβ antagonizes DOP-3 signaling (Chase *et al.* 2004). While the G proteins and other downstream signaling components of these receptor-signaling pathways in *C. elegans* are conserved and function downstream of DA receptors in mammals, how the receptor-signaling pathways functionally interact (in either organism) to modulate neural function remains largely untested.

To begin to understand how coexpressed D1- and D2-like receptors act antagonistically *in vivo*, we performed a genetic screen for DA-signaling mutants in *C. elegans*. The genes that we identified allowed us to show that signaling through coexpressed D1- and D2-like receptors oppositely modulates acetylcholine release by acting through Gαq- and Gαo-signaling pathways, respectively, and directly in the cholinergic neurons themselves.

## Materials and Methods

### Nematode culture

Worm strains were maintained at 20° under standard conditions, and double and triple mutants were generated using standard methods (Brenner 1974). Mutants analyzed in behavioral assays were as follows: *ace-2(g72) I, goa-1(sa734) I, egl-30(tg26) I, eat-16(ad702) I, cat-2(e1112) II, glr-1(nd38) III, glr-1(n2461), dat-1(ok157) III, dgk-1(sy428) X, dop-3 (vs106) X, dop-1(vs100) X*, and *ace-1(nd35) X*.

### DA resistance screen

Synchronized populations of fourth larval stage (L4) N2 animals were mutagenized with 30 μM ethyl methanesulfonate for 4 hr and cultured on NGM plates for 24 hr before $F_1$ embryos were harvested by bleach treatment of gravid adults. Synchronized L4 $F_1$ progeny were cloned to individual wells of untreated flat-bottomed 96-well plates, each well containing a culture of 50 μl of $OD_{550} = 10$ OP50 suspended in S complete media. Worm cultures were grown at 20° for 3 days in a humidified container and were then washed three times with 100 μl of water and tested for resistance to a 40-mM DA solution. A culture was scored "positive" for DA resistance if ~25% of the animals in an individual well remained thrashing in DA solution for >4 min. Resistant animals were immediately rescued from resistant cultures and cloned individually into liquid media, and their broods were again tested for DA resistance. A strain was considered homozygous if >75% of the progeny were resistant to 40 mM of liquid DA after 4 min. This screen had advantages over our previous genetic screen (Chase *et al.* 2004) that allowed us to identify additional components of DA signaling. In our previous screen, $F_2$ progeny of mutagenized animals were placed onto agar plates containing 40 mM of DA. After 20 min, rare animals were selected that were capable of spontaneous movement. The $F_2$ animals transferred to DA plates in this screen originated from a population of $F_1$ animals, and so we refer to this screen as "non-clonal" to distinguish it from our new "clonal" screen in which each group of animals tested for DA resistance is from a single $F_1$ parent. The "non-clonal" screen had two shortcomings: (1) partially resistant mutants (like *dop-3*) could not be recovered, and (2) many primary isolates did not retest (false positives).

In the current screen, $F_1$ progeny of mutagenized animals were grown individually in liquid culture microtiter plates. The $F_1$ animals are heterozygous for induced mutations and give rise to $F_2$ broods containing both mutant heterozygotes and homozygotes. Because the DA resistance test was performed on a population of $F_2$ progeny from a single $F_1$ parent in the new "clonal" screen, ~25% of the animals in a positive culture would be resistant to DA. This decreased the number of false positives that were isolated. Wells in which just a few animals were moving were considered not to be DA-resistant. Because the animals were tested for DA resistance in liquid assay rather than on agar plates, animals could be scored for resistance immediately upon exposure to DA, and thus even partially resistant mutants could be isolated.

### Mapping mutations

Mutations were mapped as described (Wicks *et al.* 2001) by mating to the polymorphic mapping strain CB4856, identifying cross-progeny, and rehomozygosing each mutation in the $F_2$ generation. Rehomozygosed mutants were identified by placing $F_2$ animals on agar plates containing 40 mM of DA and selecting animals that were resistant to paralysis after 4 min. Mutations that mapped near previously identified genes involved in DA signaling (*goa-1*, *eat-16*, and *dop-3*) were tested by standard complementation analysis using appropriate null strains.

### Transgenic animals

For rescue of DOP-3 in the cholinergic motor neurons of *dat-1; dop-3* mutants, 50 ng/μl of pCL31 (*acr-2*::GFP) and 25 ng/μl of pCL34 (*acr-2*::DOP-3) plasmids were co-injected with 15 ng/μl of pJK4 (*myo-2*::GFP). For rescue of DOP-3 in the GABAergic motor neurons of *dat-1; dop-3* mutants, 50 ng/μl of pCL32 (*unc-47*::GFP) and 25 ng/μl of pCL35 (*unc-47*::DOP-3) were co-injected with 15 ng/μl of pJK4. For rescue of DOP-3 in the cholinergic motor neurons of *dop-3 ace-1* mutants, 50 ng/μl of pCL31 and 25 ng/μl of pCL34 were co-injected with 15 ng/μl of pJK4. For rescue of DOP-1 in the cholinergic motor neurons of *dop-3 dop-1 ace-1* mutants, 50 ng/μl of pCL31 and 25 ng/μl of pCL33 (*acr-2::DOP-1*) were co-injected with 15 ng/μl of pJK4. Five independent transgenic lines were established for each experimental group, and 50 L4 animals from each line that displayed the most complete expression of GFP in the cholinergic or GABAergic motor neurons were selected and assayed for the appropriate behavior. Five control lines carrying the empty vector for each experimental condition were

generated and assayed in parallel. All transgenic lines were generated using standard methods (Mello *et al.* 1991), and all constructs were derived from the pPD49.26 vector (Addgene) using standard subcloning procedures. *acr-2* is a cholinergic neuron-specific promoter (Nurrish *et al.* 1999), and *unc-47* is a GABAergic neuron-specific promoter (Eastman *et al.* 1999). For the double transgenic animals noted in Figure 3C, an *acr-2*::GFP construct (pCL31) was coinjected with pL15EK (both at 50 ng/μl) into the strain MT8189, and GFP-positive animals were identified and mated with *unc-47*::mCherry-expressing males [strain IZ501(UfIs34), a generous gift from M. Francis]. Of the 302 neurons found in the *C. elegans* hermaphrodite, the *acr-2* promoter is active in exactly 59 neurons, 39 of which are cholinergic motor neurons in the ventral cord that express DOP-1 and DOP-3 and innervate body-wall muscles to control locomotion. The promoter is also active in six RMD motor neurons and in six IL1 sensory neurons that innervate head muscles to control foraging but not body movement and in the two PVQ interneurons. The cell bodies of these neurons can be seen in Figure 3A. The *unc-47* promoter is active in exactly 26 neurons, 19 of which are GABAergic motor neurons in the ventral cord that express DOP-3 and innervate body-wall muscles to control locomotion. The promoter is also active in four RME neurons that innervate head muscles to control foraging but not body movement, in the AVL and DVB neurons that innervate enteric muscles to control defecation, and in the RIS interneuron, ablation of which has no effect on locomotion (McIntire *et al.* 1993).

### Behavioral assays

For DA dose-response assays, ~25 young adults for each strain were incubated undisturbed for 10 min on plates containing the indicated concentration of DA and then scored for paralysis. Animals were considered paralyzed when they did not exhibit at least one spontaneous body bend in a 5-sec observation period. Assays were repeated in triplicate for a total of at least 75 animals per strain. For acute aldicarb exposure, 1-mM aldicarb plates were made by adding a 0.5-M stock solution to molten low-salt agar at 55° to a final concentration of 1 mM. Plates were stored inverted in the dark at room temperature for 24 hr and then stored at 4° and used within 1 week. Plates were allowed to equilibrate at room temperature for 30 min prior to the assay. Approximately 25 young adult animals were picked away from food and placed in the center of a 1-mM aldicarb plate and prodded every 5 min with a platinum worm pick and scored for paralysis. For Figures 5A and 5B, paralysis was defined as the inability to exhibit at least one body bend in a 5-sec period following prodding. For Figures 5C, 6A, and 6B, paralysis was defined as the inability to exhibit at least two body bends in a 5-sec period following prodding. Each assay was done in triplicate for a total of at least 75 animals per strain. Basal slowing assays were done as previously described (Chase *et al.* 2004). Briefly, the locomotion rates of staged young adult animals were quantified by counting the number of body bends completed in five consecutive 20-sec intervals in the presence or absence of HB101 bacteria. Plates with bacteria were prepared by spreading 35 μl of HB101 bacteria ($A_{600} = 0.70$–$0.75$) across each plate and incubating overnight at 37°. Data were collected for six animals per condition for a total of 30 measurements per condition. The percentage of slowing was calculated by dividing the difference between locomotion rates on and off food by the locomotion rate off food. Swimming-induced paralysis (SWIP) assays were performed by picking 10 L4 animals away from food and then placing them in a 50-μl water droplet on a Menzel Glaser 10-well diagnostic slide (#X1XER308B#) and scoring for movement after 10 min. Movement was scored as the presence of free alternating body bends characteristic of *C. elegans* swimming behavior (Pierce-Shimomura *et al.* 2008). In the case of locomotion-defective mutants, movement was scored as the continual exhibition of spontaneous body bends. This assay was repeated for a total of 50 animals per strain.

### Statistical analyses

Comparisons shown in Figure 2B, Figure 3B, and Figure 4 were done using two-tailed Student's *t*-test. In Figure 2A, error bars represent the average of three trials and 95% confidence intervals; in Figure 5 and Figure 6, error bars represent the means of three trials and SEM. In these figures, we compared the curves of each mutant to the wild-type or other appropriate control (see below) using a two-way ANOVA with repeated measures followed by a Bonferroni multiple comparisons post hoc test. In Figure 2A, the curves of all mutants were statistically different from WT at multiple DA concentrations [*ace-1(nd35)* at 15, 20, 30, and 40 mM, $P < 0.0001$; *dop-3(vs106)*, *glr-1(nd38)*, and *glr-1(A/T)* at 15, 20, 30, 40, 60, and 80 mM, $P < 0.0001$]. For Figure 5A, the curves for *dop-3(vs106)* and *ace-1(nd35)* mutants were not statistically different from WT ($P > 0.05$), but the curves for *glr-1(nd38)* and *glr-1(A/T)* animals were: *glr-1(nd38)* (at $t = 35$–$70$ min; $P < 0.0001$) and *glr-1(A/T)* (at $t = 40$–$70$ min; $P < 0.0001$). For Figure 5B, the curve for *glr-1(nd38); dop-3(vs106)* double mutants was statistically different from that for *glr-1(nd38)* animals (at $t = 30$, 35, 40, and 45 min; $P < 0.0001$). In Figure 5C, the curve for *dop-3(vs106) ace-1(nd35)* double mutants was statistically different from that for *ace-1(nd35)* and *dop-3(vs106)* single mutants (at $t = 25$ min, $P < 0.001$, and at $t = 30$–$60$ min, $P < 0.0001$). In Figure 6A, we compared the curve for *dop-3(vs106) ace-1(nd35)* double mutants expressing the empty transgene to that for *dop-3(vs106) ace-1(nd35)* double mutants expressing the *acr-2::DOP-3* rescuing transgene and found that they were different (at $t = 20$ min, $P < 0.001$, and at $t = 25$–$65$ min, $P < 0.0001$). In Figure 6B, we compared the curve for the *dop-1(vs100) dop-3(vs106) ace-1(nd35)* triple mutant expressing the empty transgene with the triple mutant expressing the *acr-2::DOP-1* rescuing transgene and found that they were different (at $t = 20$ min, $P < 0.001$, and at $t = 25$–$65$ min, $P < 0.0001$).

**Table 1 Genes and mutations identified in the dopamine resistance screen**

| Gene | Alleles | Protein | Human ortholog |
|---|---|---|---|
| *goa-1* | *nd64* | G-protein $\alpha$-subunit | $G\alpha_o$ |
| *dgk-1* | *nd48, nd55* | Diacylglycerol kinase | DGK$\theta$ |
| *egl-30* | *nd50* | G-protein $\alpha$-subunit | $G\alpha_q$ |
| *dop-3* | *nd41* | D2-like receptor | D2 receptor |
| *glr-1* | *nd38* | AMPA-type glutamate-gated cation channel subunit | GluRd2 |
| *ace-1* | *nd35* | Class A acetylcholinesterase | Acetylcholinesterase |

## Results

### *Genetic screen for DA-resistant mutants*

In *C. elegans*, DA acts through the D2-like receptor DOP-3 to inhibit locomotion rate in response to the presence of food, allowing animals to remain in a food-rich environment (Chase *et al.* 2004). Exogenous DA also inhibits locomotion, high concentrations cause paralysis, and *dop-3* receptor mutants are resistant to this paralysis (Schafer and Kenyon 1995; Chase *et al.* 2004). In a previous genetic screen for mutants resistant to paralysis, we identified some components of endogenous DA signaling; however, we did not identify all components since, for example, mutations in the DOP-3 receptor were not obtained (Chase *et al.* 2004). Here we performed a more sensitive genetic screen for weakly resistant mutants and isolated seven DA-resistant mutants that identify additional genes involved in DA response not identified in our previous screen.

We mapped the mutations and used complementation tests and DNA sequence analysis to show that the seven mutations identified six genes (Table 1). Mutations in *goa-1, dgk-1*, and *dop-3* were loss-of-function mutations, and the *egl-30* mutation was a gain-of-function mutation (data not shown). The proteins encoded by these four genes are components of two G protein-signaling pathways that we previously showed act antagonistically to mediate DA's control of locomotion in *C. elegans* (Figure 1A and Chase *et al.* 2004). The DOP-3 receptor is coupled to the G protein G$\alpha$o/GOA-1 to inhibit locomotion; signaling through this pathway is terminated by the GTPase activity of the regulator of G protein-signaling (RGS) protein EGL-10 and the G$\beta_5$ protein GPB-2. Signaling by DOP-3 is antagonized by signaling through the DOP-1 receptor, which is coupled to the G protein G$\alpha$q/EGL-30. G$\alpha$q/EGL-30 activates phospholipase C$\beta$ (PLC$\beta$/EGL−8) to generate diacylglycerol and inositol trisphosphate from phosphotidylinositol 4,5-bisphosphate. G$\alpha$q/EGL-30 signaling is terminated by the RGS protein EAT-16 and G$\beta_5$/GPB-2 (Chase *et al.* 2004).

### *Mutations in two other genes (*glr-1 *and* ace-1*) identified in the genetic screen are predicted to increase acetylcholine signaling from ventral-cord motor neurons*

We mapped mutations in two other genes not identified in our previous screen. The first of these mutations, *nd38*, was in the gene encoding the AMPA-type glutamate receptor subunit GLR-1 (Figure 1B). GLR-1 expression is largely restricted to 10 command interneurons—two each of AVD, AVA, AVE, AVB, and PVC—which receive input from sensory neurons and innervate ventral-cord cholinergic motor neurons to control forward and backward locomotion (Chalfie *et al.* 1985; Brockie *et al.* 2001) (Figure 1C). In addition to causing DA resistance, the *glr-1(nd38)* mutation caused animals to reverse their direction of movement at a dramatically higher frequency (36.4 ± 0.3 reversals/min) than wild-type animals (2.1 ± 0.1 reversals/min), and this effect was dominant [*glr-1 (nd38)/+* heterozygotes reversed 35.6 ± 0.3 times/min]. Similarly increased reversal frequencies were caused by the expression of a transgene containing a dominant mutation in GLR-1 [*glr-1(A/T)*], designed to mimic the mutation found in the homologous δ2 glutamate receptor subunit of the "Lurcher" mutant mouse (Zuo *et al.* 1997; Zheng *et al.* 1999) (Figure 1B). Both the *glr-1(A/T)* and the mutant δ2 glutamate receptor subunits caused increased ion conductance when expressed in Xenopus oocytes (Zuo *et al.* 1997; Zheng *et al.* 1999), and the mutant δ2 glutamate receptor subunit caused neurodegeneration of cerebellar Purkinje cells *in vivo* (Zuo *et al.* 1997), suggesting that the Ala/Thr mutation caused the mutant ion channel to be constitutively active.

The *glr-1(nd38)* mutation isolated in our genetic screen caused a serine-to-proline change (S668P) in the third transmembrane domain (TMDIII) of GLR-1, which is very near the position of the amino acid mutated in the Lurcher channel (Figure 1B). TMDIII helices of AMPA glutamate receptor subunits line the inside surface of the channel and physically cross each other to block the ion channel in its resting, closed state (Sobolevsky *et al.* 2009). The introduction of a proline residue within TMDIII would confine the position of the TMDIII helix, potentially disrupting the crossing of the TMDIII helices and resulting in a constitutively open ion channel. Because the *glr-1(nd38)* mutation is in the TMDIII helix of the channel, like the Lurcher mutation and because it causes dominant effects on locomotion as the GLR-1 A/T transgene does, we suggest that the *glr-1(nd38)* mutation is a gain-of-function mutation that causes constitutive activity of the GLR-1 channel and hyperactivity of the command interneurons. Since the command interneurons innervate only cholinergic motor neurons (White *et al.* 1976), it is likely that the *glr-1(nd38)* mutation causes increased stimulation of the cholinergic motor neurons and increased

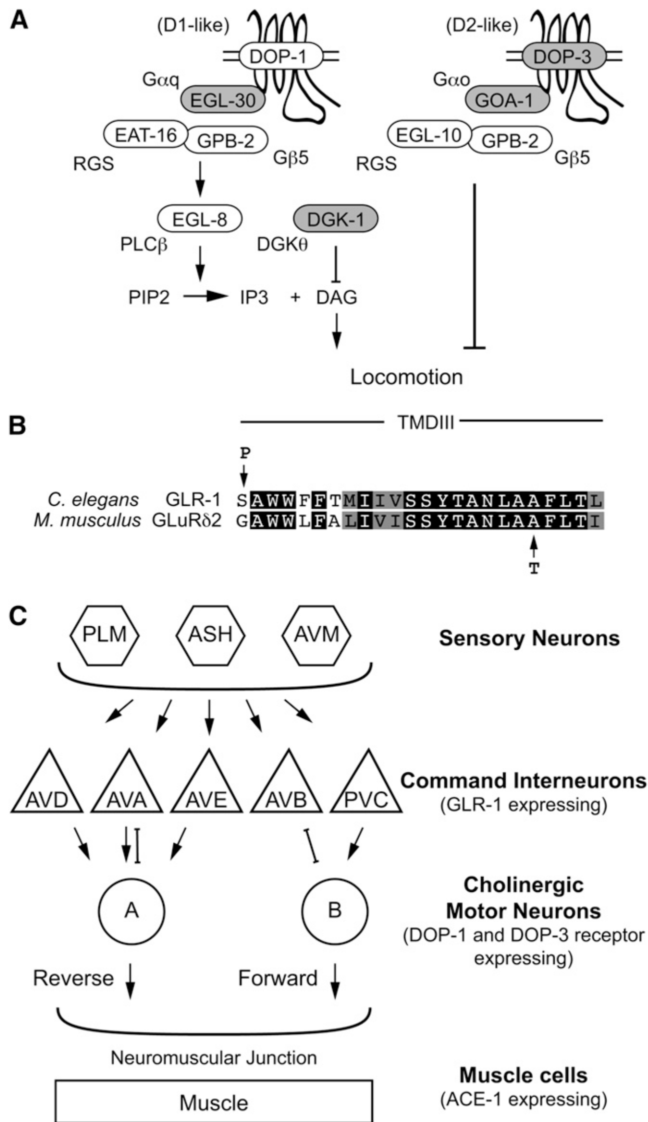


**Figure 1** Mutations and signaling pathways that control locomotion in *C*. elegans. (A) Schematic of the opposing dopamine-signaling pathways that act to modulate locomotion behavior in *C. elegans*. Dopamine inhibits locomotion by binding to DOP-3 and activating Gαo signaling, while dopamine enhances locomotion by binding to DOP-1 and activating Gαq signaling. The G protein-signaling components identified in our genetic screen are indicated by shaded ovals. Names of *C. elegans*-signaling proteins are shown inside the ovals and names of their mammalian homologs are shown outside the ovals. (B) Alignment of the third transmembrane domain region (TMDIII) of AMPA glutamate receptors from *C. elegans* and mammals. The residues mutated in *glr-1(nd38)* and the Lurcher mouse are indicated. A solid background indicates identical residues; a shaded background indicates chemically similar residues. (C) Schematic of the neural circuitry in *C. elegans* that controls locomotion behavior. Many sensory neurons, including PLM, ASH, and AVM, innervate the command interneurons (AVD, AVA, AVE, AVB, and PVC), which integrate these signals and activate the cholinergic motor neurons to control forward and backward locomotion. GLR-1 expression is largely restricted to the command interneurons, the DOP-1 and DOP-3 receptors are coexpressed in the cholinergic motor neurons, and ACE-1 is expressed in the muscle cells. Arrows indicate chemical synapses between the indicated cell types and bars indicate gap junctions.

acetylcholine release into the neuromuscular junction (NMJ), resulting in the hyper-reversal phenotype.

The second mutation, *nd35*, was a nonsense mutation (Q84STOP) in the class A acetylcholinesterase *ace-1*. ACE-1 is released from muscle cells into the neuromuscular junction where it terminates cholinergic signaling by degrading acetylcholine (Herman and Kari 1985) (Figure 1C). Thus, like the *glr-1(nd38)* mutation, *ace-1(nd35)* should increase acetylcholine levels in the neuromuscular junction.

#### glr-1(nd38) *and* ace-1(nd35) *mutations cause defects in DA signaling*

To quantify the DA-signaling defect of *glr-1(nd38)* and *ace-1 (nd35)* mutants, we examined their response to exogenous DA (Figure 2A). Wild-type animals show a dosage-dependent decrease in locomotion upon exposure to DA and are completely paralyzed by exposure to 40 mM DA (Schafer and Kenyon 1995; Chase *et al.* 2004) (Figure 2A). Such high concentrations of DA are required because the *C. elegans* cuticle acts as a permeability barrier to drugs (Lewis *et al.* 1980). Mutations in DOP-3 caused partial resistance to DA. At 40 mM DA, >80% of *dop-3(vs106)* mutants were capable of movement, whereas none of the wild-type animals moved (Figure 2A). We found that *ace-1(nd35)* mutants were also resistant to exogenous DA (Figure 2A). The facts that exogenous DA causes paralysis by activating DOP-3 receptors expressed in the cholinergic motor neurons (Chase *et al.* 2004) and the only known function of ACE-1 is to degrade acetylcholine in the NMJ suggest that the physiological role of DOP-3 receptor signaling in the motor neurons is to inhibit acetylcholine release into the NMJ.

*glr-1(nd38)* mutants were more resistant to exogenous DA than either *dop-3(vs106)* or *ace-1(nd35)* mutants (Figure 2A). To support the idea that *glr-1(nd38)* represents a gain-of-function mutation, we tested *glr-1(A/T)* transgenic animals for defects in dopamine response and found that they were resistant to exogenous dopamine-like *glr-1(nd38)* mutants (Figure 2A). Since GLR-1 is expressed in the command interneurons that innervate the DA-receptive cholinergic motor neurons (White *et al.* 1976; Brockie *et al.* 2001), this result suggests that, if DA does inhibit acetylcholine release from the motor neurons under physiological conditions, its inhibitory effects are limited as exogenous DA cannot block excess neural activity caused by hyperactivity of the upstream command interneurons.

From these results we propose a model in which locomotion behavior in *C. elegans* is modulated by DA's control of acetylcholine release from the cholinergic motor neurons into the neuromuscular junction. This model predicts that mutations that cause abnormal acetylcholine release from these neurons should cause defects in endogenous DA-specific locomotion behaviors. Thus we examined *ace-1 (nd35)* and *glr-1(nd38)* mutants for defects in basal slowing behavior. Well-fed, wild-type animals slow their locomotion rate when they encounter a bacterial food source, and this "basal slowing" is controlled by DA signaling (Sawin *et al.*

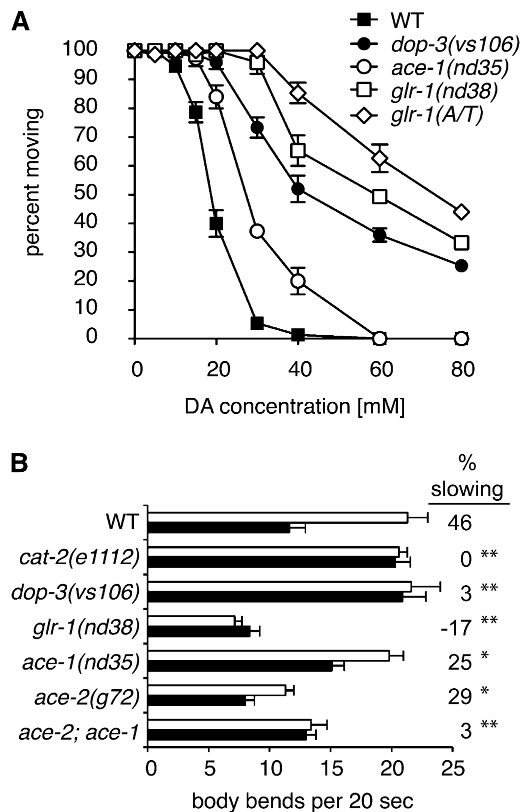


**Figure 2** Analysis of dopamine-signaling defects in *ace-1(nd35)* and *glr-1(nd38)* mutants. (A) Dose-response curves measuring paralysis induced by exogenous dopamine. Shown is the percentage of animals moving 10 min after being placed on agar plates containing the indicated concentrations of dopamine. Each data point represents the mean ± SEM for three trials totaling at least 75 animals. (B) Quantitative analysis of basal slowing behavior. For each strain, locomotion rates in the absence of bacteria (open bars) and the presence of bacteria (solid bars) were calculated as the average of 30 observations. Error margins shown indicate 95% confidence intervals. Asterisks indicate values significantly different from the 46% slowing seen in the wild type (Student's *t*-test: $*P < 0.01$, $**P < 0.0001$). The percentage slowing in the presence of bacteria for each strain is shown at the right.

2000). *dop-3(vs106)* and *cat-2(e1112)* mutants (*cat-2* encodes tyrosine hydroxylase) fail to slow in response to food (Sawin *et al.* 2000; Chase *et al.* 2004) (Figure 2B). We found that both *glr-1(nd38)* and *ace-1(nd35)* mutants were defective in basal slowing (Figure 2B). *glr-1(nd38)* mutants were completely defective in slowing while *ace-1(nd35)* mutants showed a more modest defect. We tested another allele of *ace-1* [*ace-1(p1000)*] and found that it, too, caused significant defects in slowing behavior (data not shown). We note that, while *glr-1(nd38)* mutants appear to move slower than wild-type animals in this assay, the reduced locomotion is not due to slower movement but rather to the frequent reversal behavior of *glr-1(nd38)* mutants, which often occurs before an animal can achieve a full body bend resulting in lower locomotion assay scores in both the presence and the absence of food.

If DA inhibits acetylcholine release from motor neurons in response to food to cause slowing, why are *ace-1* null mutants only partially defective in slowing? *C. elegans* contains three other acetylcholinesterases: *ace-2, ace-3*, and *ace-4* (Combes *et al.* 2003). However, *ace-4* encodes a divergent acetylcholinesterase with no detectable biochemical activity, and *ace-3* expression is restricted to pharyngeal muscles (Combes *et al.* 2003). In contrast, *ace-2* is expressed in the ventral-cord motor neurons, and so we tested *ace-2(g72)* null mutants for defects in basal slowing. We found that, like *ace-1(nd35)* mutants, *ace-2(g72)* mutants were partially defective in slowing (Figure 2B). However, we found that *ace-2(g72); ace-1(nd35)* double-mutant animals were completely defective like *dop-3(vs106)* and *cat-2(e1112)* mutants (Figure 2B). This suggests that slowing in response to food is caused by DA acting through the DOP-3 receptor to inhibit acetylcholine release from the motor neurons.

#### *A behavior mediated by endogenous DA, swimming-induced paralysis, is caused by increased DA signaling specifically in the ventral-cord motor neurons*

While we have shown that basal slowing requires acetylcholine signaling and is modulated by endogenous DA, we have not formally shown that the modulation of acetylcholine signaling by DA occurs in the cholinergic motor neurons. One could investigate the cellular locus of basal slowing using transgene rescue experiments, but the basal slowing response is modest (46% slowing; Figure 2B) and thus not well suited to transgenic rescue experiments. Therefore, we investigated the site of action of another, more robust, DA-specific locomotion behavior: swimming-induced paralysis. Wild-type animals placed in water swim vigorously for >30 min while animals with increased DA signaling caused by mutation of the DA reuptake transporter DAT-1 become paralyzed within 10 min (McDonald *et al.* 2007) (Figure 3). Swimming-induced paralysis is a robust behavioral phenotype as >80% of wild-type animals continue to swim after 10 min while nearly 100% of *dat-1(ok157)* animals are paralyzed at this time. The excess DA present in *dat-1(ok157)* mutants acts through DOP-3 to cause SWIP as *dat-1(ok157); dop-3(vs106)* double mutants do not show SWIP (McDonald *et al.* 2007) (Figure 3 and supporting information movies, File S1, File S2, and File S3 ).

To determine whether SWIP is controlled by DA signaling in the ventral-cord motor neurons, we used promoters active in the cholinergic and GABAergic neurons of the ventral cord to express DOP-3 in these cells and tested the ability of such transgenes to reverse the suppression of SWIP seen in *dat-1(ok157); dop-3(vs106)* double mutants (Figure 3A). Wild-type and control strains were tested for SWIP (Figure 3). As previously reported, wild-type animals and *dop-3(vs106)* mutants swam vigorously for >30 min when placed in water while *dat-1(ok157)* mutants became paralyzed within 10 min (McDonald *et al.* 2007) (Figure 3B). The *dop-3(vs106)* mutation suppressed the SWIP phenotype of *dat-1(ok157)* mutants as *dat-1(ok157); dop-3(vs106)* double mutants swam freely like wild-type animals (Figure 3B). To test whether SWIP was caused by DA signaling through

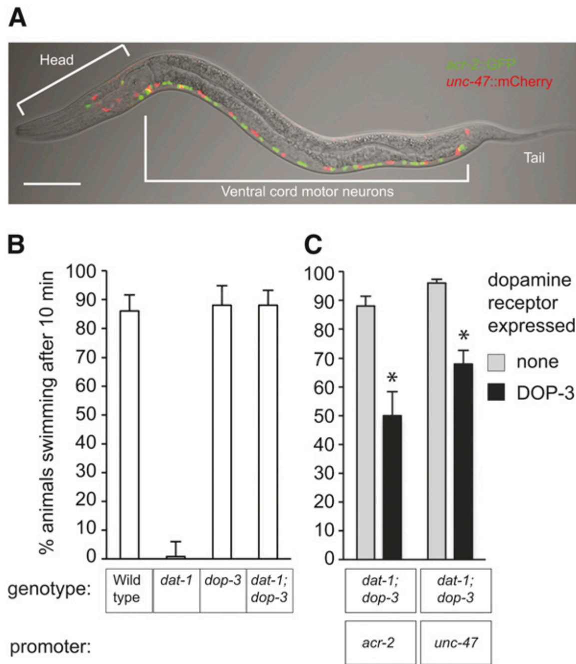


**Figure 3** Quantitative analysis of swimming-induced paralysis. Swimming-induced paralysis is mediated by DOP-3 signaling in the ventral-cord motor neurons. (A) Photomicrograph of a double-transgenic animal in which green fluorescent protein (GFP) is expressed in the cholinergic motor neurons using the *acr-2* promoter, and red fluorescent protein (mCherry) is expressed in the GABAergic motor neurons using the *unc-47* promoter (bottom bracket). A few other neurons located in the head (top bracket) express GFP or mCherry, but these neurons do not innervate body-wall muscles to control locomotion. Scale bar, 20 μm. (B) SWIP behavior of control nontransgenic strains. Each measurement shown represents the mean of five trials of 10 L4 animals each for a total of 50 animals. Error bars represent 95% confidence intervals. (C) SWIP behavior of *dat-1; dop-3* double mutants carrying transgenes. The promoters used for transgenic expression are indicated at the bottom. Shaded bars represent measurements from control strains carrying empty vector transgenes, which have promoters but no receptor sequences. Solid bars represent measurements from strains carrying transgenes from which the promoters express the DOP-3 receptor. For each transgene, measurements of 50 animals for each of five independent transgenic lines were averaged, and the means and 95% confidence intervals are shown. An asterisk indicates that receptor expression gave significant rescue compared to the control (unpaired Student's *t*-test: $P < 0.0001$). Both the *acr-2* and the *unc-47* promoters gave significant rescue, indicating that swimming-induced paralysis is caused, at least in part, by dopamine acting through DOP-3 receptors expressed in both the cholinergic and the GABAergic motor neurons.

the DOP-3 receptor in cholinergic motor neurons, we expressed the DOP-3 receptor from the *acr-2* promoter, which is active in the cholinergic motor neurons of the ventral cord (Nurrish *et al.* 1999). Expression of the *acr-2*::DOP-3 transgene in *dat-1(ok157); dop-3(vs106)* double mutants partially rescued *dat-1*-dependent SWIP, such that only 50% of animals were moving compared to 88% for empty vector controls (Figure 3C). Because the DOP-3 receptor is also expressed in the GABAergic motor neurons (Chase *et al.* 2004), we expressed the DOP-3 receptor in the GABAergic neurons using the *unc-47* promoter (Eastman *et al.* 1999). This also resulted in partial rescue of paralysis (68% of animals moving compared to 96% for empty vector control). Thus, SWIP is, at least in part, caused by endogenous DA acting through the DOP-3 receptor in both the cholinergic and the GABAergic ventral-cord motor neurons.

### *SWIP is mediated by antagonistic signaling through D1 and D2 receptors*

We next tested whether SWIP is caused by DA acting through the antagonistic D1/D2-signaling mechanism that we identified previously (Figure 1A). Thus we tested *dop-1(vs100)* and *dop-3(vs106)* single- and *dop-1(vs100) dop-3(vs106)* double-receptor mutants for SWIP. While the *dop-3(vs106)* mutation suppressed the SWIP phenotype of *dat-1(ok157)* mutants, the *dop-1(vs100)* mutation could not (Figure 4, A and B). Interestingly, *dat-1(ok157); dop-1(vs105) dop-3(vs106)* triple mutants showed a SWIP phenotype intermediate between that of *dat-1(ok157)* and *dat-1(ok157); dop-3(vs106)* double mutants [<1% moving for *dat-1(ok157)*, 72% moving for *dat-1(ok157); dop-1(vs105) dop-3(vs106)* triple mutant, and 88% moving for *dat-1(ok157); dop-3(vs106)* double mutants], suggesting that SWIP is mediated by signaling through both D1 and D2 receptors and that the two receptors have opposite effects on SWIP (Figure 4B). We next tested other components of the D1/D2 receptor-signaling pathways, including *goa-1(sa734)*, *eat-16(ad702)*, and *dgk-1(sy428)* null mutants and *egl-30(tg26)* gain-of-function mutants (Figure 1A), and found that mutations that either reduced signaling through the DOP-3 receptor (including mutations in the DOP-3 receptor and the Gαo/GOA-1 G protein α-subunit) or increased signaling through the DOP-1 receptor (including gain-of-function mutations in Gαq/EGL-30 and loss-of-function mutations in RGS/EAT-16 and DGKθ/DGK-1) could suppress the SWIP phenotype of *dat-1(ok157)* mutants (Figure 4C), demonstrating that endogenous DA acts through the antagonistic D1/D2-signaling pathways shown in Figure 1A to mediate SWIP.

### glr-1(nd38) *and* ace-1(nd35) *mutants also suppress* dat-1*-induced SWIP*

If SWIP is caused by reduced acetylcholine release from the cholinergic motor neurons in *dat-1(ok157)* mutants (caused by excess DA signaling through DOP-3), one would expect that the *glr-1(nd38)* and *ace-1(nd35)* mutations would also suppress the SWIP phenotype. Indeed, we found that, while *glr-1(nd38)* and *ace-1(nd35)* single mutants were able to swim like wild-type animals, both mutations suppressed the SWIP phenotype of *dat-1(ok157)* mutants (Figure 4D). While 0.8% of *dat-1(ok157)* animals can swim, 56% of *dat-1(ok157); glr-1(nd38)* and 44% of *dat-1(ok157); ace-1(nd35)* animals were still swimming after 10 min. Thus, mutations that increase acetylcholine signaling in the neuromuscular junction suppress SWIP.

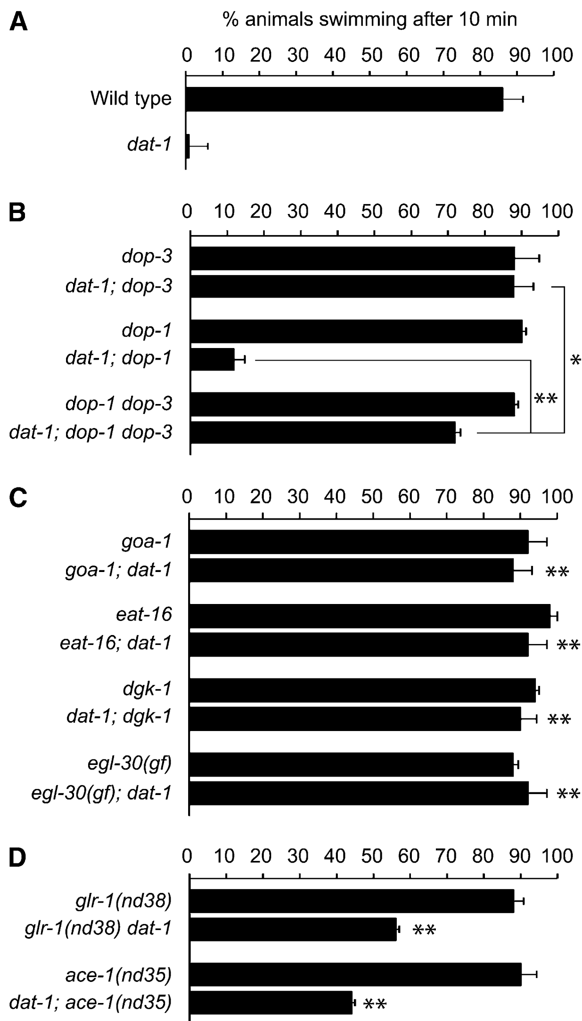


**Figure 4** Quantitative analysis of SWIP behavior of dopamine-signaling mutants. (A–D) Each measurement represents the mean of five trials of 10 L4 animals each for a total of 50 animals per strain. Error bars represent 95% confidence intervals. (A) *dat-1* mutants are paralyzed compared to wild-type animals. (B) Mutations in the DOP-1 and DOP-3 receptors have opposite effects on SWIP. (C) All dopamine-signaling mutants predicted to increase acetylcholine signaling in the neuromuscular junction suppress *dat-1* swimming-induced paralysis. (D) *glr-1(nd38)* and *ace-1(nd35)* mutations suppress SWIP. Asterisks in C and D indicate a significant difference from *dat-1* mutants (Student's *t*-test: $**P < 0.0001$). Asterisks in B indicate significant difference between the *dat-1; dop-1 dop-3* triple mutant and the double mutants indicated (Student's *t*-test: $*P < 0.05$, $**P < 0.0001$).

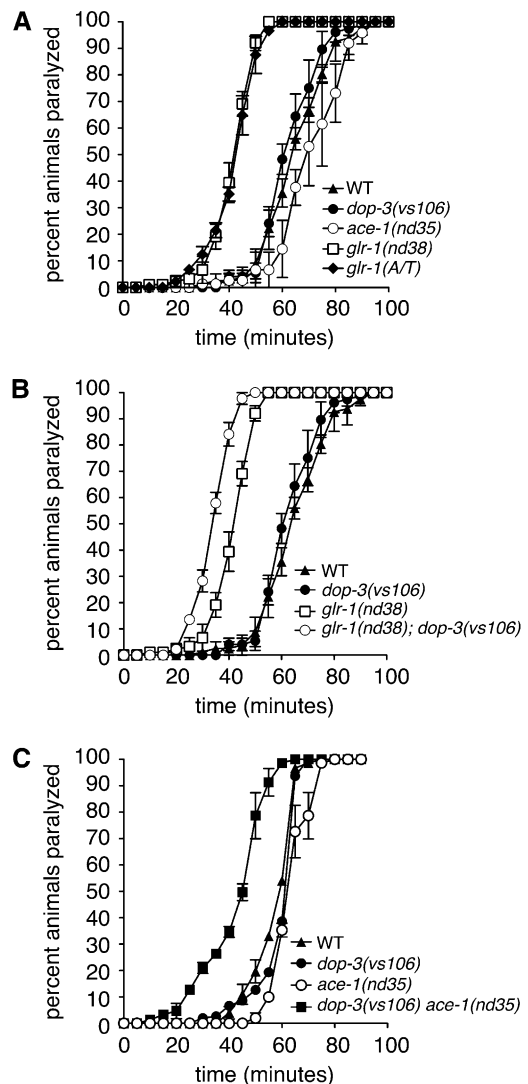


**Figure 5** *dop-3(vs106)*, *ace-1(nd35)*, and *glr-1(nd38)* mutations all cause increased acetylcholine release into the neuromuscular junction. (A–C) Quantitative analysis of acetylcholine release into the neuromuscular junction. Shown is the percentage of animals paralyzed at the indicated times after being placed on agar plates containing 1 mM of aldicarb. Each data point represents the mean ± SEM for three trials totaling at least 75 animals.

### dop-3(vs106), ace-1(nd35), *and* glr-1(nd38) *mutations cause increased acetylcholine release from ventral-cord motor neurons*

To determine if DA modulates acetylcholine release from the ventral-cord motor neurons, we measured the sensitivity of mutants to the acetylcholinesterase inhibitor aldicarb (Figure 5). Aldicarb increases the concentration of acetylcholine in the neuromuscular junction by inhibiting its degradation, causing muscle contraction and a time-dependent paralysis of treated animals. Mutations that cause reduced acetylcholine release, such as mutations in the synaptic vesicle protein synaptobrevin (*snb-1)*, cause resistance to aldicarb-induced paralysis (Nonet *et al.* 1998), and mutations that cause increased acetylcholine release, such as mutations in an inhibitor of synaptic vesicle priming tomosyn *(tom-1)*, are hypersensitive to aldicarb (Dybbs *et al.* 2005). We found that *glr-1(nd38)* mutants were hypersensitive to aldicarb (Figure 5A). We also tested *glr-1(A/T)* gain-of-function animals and found that they were also hypersensitive to aldicarb, again suggesting that *glr-1(nd38)* is a gain-of-function mutation. However, we were surprised to find that both *ace-1(nd35)* and *dop-3(vs106)* mutants were not hypersensitive to aldicarb treatment (Figure 5A).

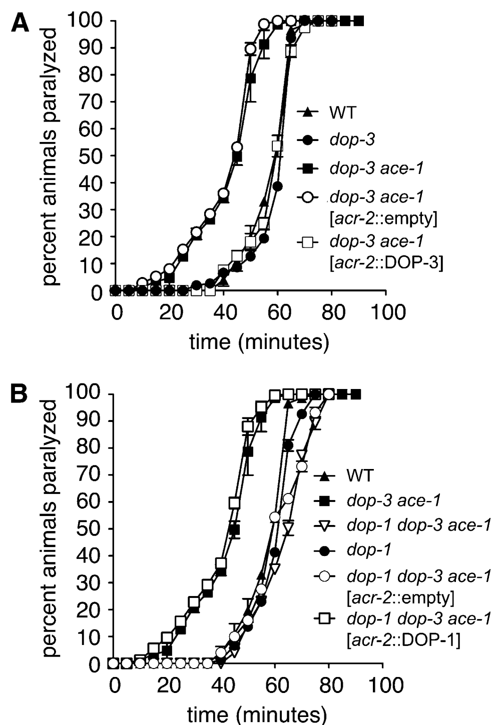


**Figure 6** DOP-1 and DOP-3 receptors act antagonistically in the cholinergic motor neurons to modulate acetylcholine release. (A and B) Quantitative analysis of acetylcholine release into the neuromuscular junction. Shown is the percentage of animals paralyzed at the indicated times after being placed on agar plates containing 1 mM of aldicarb. Each data point for nontransgenic animals represents the mean ± SEM for three trials totaling at least 75 animals. For transgenic animals, each data point represents the average of 250 animals (two trials of 25 animals per line and a total of five lines per transgene). (A) The DOP-3 receptor acts to inhibit acetylcholine release from the cholinergic motor neurons. (B) The DOP-1 receptor acts to enhance acetylcholine release from the cholinergic motor neurons.

Because ACE-1 is released into the neuromuscular junction, the aldicarb sensitivity of *ace-1(nd35)* mutants suggested that the aldicarb assay might not be sufficiently sensitive to detect subtle changes in acetylcholine signaling. We tested *ace-2(g72)* mutants and found they were sensitive to aldicarb (data not shown). We then combined the *ace-1(nd35)* and *ace-2(g72)* mutations and found that *ace-2(g72); ace-1(nd35)* double mutants were more sensitive to aldicarb than the *ace-2(g72)* single mutants (data not shown), indicating that both acetylcholinesterases function in the neuromuscular junction to degrade acetylcholine. This result suggested that the wild-type aldicarb sensitivity of *dop-3(vs106)* mutants might also be due to a lack of sensitivity of the assay. Thus we combined the *dop-3 (vs106)* mutation with mutations in *ace-1(nd35)* and *glr-1 (nd38)* (Figure 5, B and C). We found that *glr-1(nd38); dop-3(vs106)* and *dop-3(vs106) ace-1(nd35)* double mutants were more sensitive to aldicarb than either *glr-1(nd38)* or *ace-1(nd35)* single mutants, respectively. The weak effects of *dop-3(vs106)* mutations in the aldicarb response assays might be caused by two factors. First, aldicarb assays are performed on agar plates without food, and DOP-3 signaling is stimulated when an animal encounters food (and thus DA levels are presumably low in the absence of food) (Sawin *et al.* 2000). Second, electrophysiological recordings in mammals consistently show that D2 receptor signaling does not block neurotransmission but rather causes a 20–30% reduction in neural activity (*e.g.*, Surmeier *et al.* 1996). Such modest changes in excitability have dramatic physiological consequences but may be below the sensitivity of the aldicarb assay. Regardless, our results clearly indicate that DA signaling through DOP-3 inhibits acetylcholine release from the cholinergic motor neurons.

### *DOP-1- and DOP-3-receptor signaling have opposite effects on acetylcholine release from the ventral-cord motor neurons*

To demonstrate that DOP-3 acts in the cholinergic motor neurons to inhibit acetylcholine release, we used the *acr-2* promoter to express DOP-3 back in the cholinergic motor neurons and test the ability of this transgene to rescue the effects of *dop-3(vs106)* mutations on aldicarb hypersensitivity. Because the only defects caused by the *dop-3(vs106)* mutation were observed in the *ace-1(nd35)* [or *glr-1 (nd38)*] mutant background, we tested for the ability of the *acr-2*::DOP-3 transgene to reduce the aldicarb hypersensitivity of the *dop-3(vs106) ace-1(nd35)* double mutant back to the aldicarb sensitivity of *ace-1(nd35)* mutants. Indeed, the *acr-2*::DOP-3 transgene rescued the *dop-3(vs106) ace-1 (nd35)* double mutant back to wild-type [and *ace-1(nd35)*] sensitivity (Figure 6A). Thus DOP-3 functions in the cholinergic motor neurons to inhibit acetylcholine release.

We next tested whether DA acted antagonistically through coexpressed D1 and D2 receptors to modulate acetylcholine release from the motor neurons. We tested this in two ways. First, we compared the aldicarb sensitivity of *dop-1(vs100) dop-3(vs106) ace-1(nd35)* triple mutants to *dop-3(vs106) ace-1(nd35)* double mutants. While *dop-3 (vs106) ace-1(nd35)* double mutants were hypersensitive to aldicarb, the triple mutant had aldicarb sensitivity similar to wild-type animals [and *dop-1(vs100)*, *dop-3(vs106)*, and *ace-1(nd35)* single mutants], indicating that the *dop-1 (vs100)* and *dop-3(vs106)* mutations had opposite effects on acetylcholine release (Figure 6B). To demonstrate that the opposite effects on aldicarb sensitivity were due to the antagonistic function of DOP-1 receptors in the cholinergic motor neurons, we used the *acr-2* promoter to express DOP-1 back in the cholinergic motor neurons and tested the ability of this transgene to rescue the effects of the *dop-1(vs100)* mutation on aldicarb sensitivity. Thus we tested the ability of the *acr-2*::DOP-1 transgene to increase the aldicarb sensitivity of *dop-1(vs100) dop-3(vs106) ace-1(nd35)* triple mutants back to that seen in *dop-3(vs106) ace-1(nd35)* double mutants. We found that *dop-1(vs100) dop-3(vs106) ace-1 (nd35)* triple mutants carrying the *acr-2*::DOP-1 transgene were indistinguishable from *dop-3(vs106) ace-1(nd35)* double mutants (Figure 6B). Thus DA acts through coexpressed

D1 and D2 receptors in the cholinergic motor neurons of *C. elegans* to mediate acetylcholine release. Signaling through the D1 receptor enhances acetylcholine release, and signaling through the D2 receptor inhibits acetylcholine release.

## Discussion

In our previous analysis of DA signaling in *C. elegans* (Chase *et al.* 2004), we determined the expression pattern of the DOP-1 and DOP-3 receptors and showed that they acted antagonistically to modulate locomotion behavior. We did not identify the neurons through which endogenous DA controlled locomotion, but we did show that exogenous DA caused paralysis by hyper-activating DOP-3 receptors expressed on cholinergic and GABAergic motor neurons. Exogenous DA also acted through DOP-1 receptors in the cholinergic motor neurons to enhance locomotion, but this effect was masked by the overriding inhibitory effects of DOP-3 signaling. In a genetic screen for mutants resistant to the paralytic effects of exogenous DA, we identified endogenous signaling components and pathways used by DA (Figure 1A).

We have extended our analysis of DA signaling to show that endogenously released DA controls locomotion by acting through DOP-1 and DOP-3 receptors that are coexpressed on cholinergic motor neurons. DOP-3 signaling in these cells inhibits acetylcholine release, and DOP-1 signaling enhances acetylcholine release in these same cells.

### *D1- and D2-like receptors are also coexpressed on neurons in the mammalian brain*

We suspect the antagonistic mechanism of DA signaling that we have identified in *C. elegans* is conserved in the mammalian brain as there are many similarities between DA signaling in the nervous systems of these two organisms, including the coexpression of DA receptor subtypes.

In *C. elegans*, DOP-1 and DOP-3 receptors are coexpressed in the cholinergic motor neurons that control locomotion. In medium spiny neurons of the mammalian striatum, D1 and D2 receptors are largely segregated to different neuron populations; however, about 20% of medium spiny neurons express both D1 and D2 receptors (Gerfen *et al.* 1990; Le Moine and Bloch 1995; Surmeier *et al.* 1996; Bertran-Gonzalez *et al.* 2008). If one considers all five DA receptor subtypes, the overlap in expression between two or more receptor subtypes increases to 20–50% of all striatal medium spiny neurons (Surmeier *et al.* 1996). Nearly 90% of all aspiny cholinergic neurons of the striatum coexpress at least one D1-like class and one D2-like class receptor (Yan and Surmeier 1997).

While it is clear that D1- and D2-like receptors are coexpressed in at least some classes of neurons in the brain, few studies have investigated how such coexpressed receptors interact functionally *in vivo*. Recent work, however, has suggested that D1 and D2 receptors physically interact in striatal neurons to form heterooligomers that couple to the activation of Gαq (Rashid *et al.* 2007). In this case, activation of Gαq signaling is specific to D1/D2 heterooligomers as dopamine or co-application of agonists against both receptor classes is necessary to elicit Gαq activation and removal of either receptor by mutation eliminated Gαq signaling (Rashid *et al.* 2007). It is formally possible that the Gαq activation that we observe in *C. elegans* and attribute to the DOP-1 receptor is the result of signaling through such a D1/D2 heterooligomer. However, such a heterooligomer would almost certainly not contain DOP-3. If DOP-1 and DOP-3 functioned as a heterooligomer, one would predict that mutation of either receptor would disrupt Gαq signaling and acetylcholine release. Instead, we found that mutations in *dop-1* and *dop-3* have opposite effects on acetylcholine release.

In other experiments, Surmeier *et al.* (1992) measured the response of single, acutely isolated rat striatonigral neurons to both D1-like and D2-like agonists and found that signaling through both receptor types separately reduced the amplitude of evoked sodium currents. Agonists against both receptor classes were not applied simultaneously, and so functional interactions between the receptors were not evaluated. In these cells, signaling through D1 and D2 receptors had similar effects on activity. This result is different from the antagonistic effects that we observe between DOP-1 and DOP-3 receptors in *C. elegans* motor neurons and could be the result of one of several factors. First, it was not clear that the neurons examined by Surmeier's group expressed only one D1-like or one D2-like receptor. Second, the dendritic and axonal projections of isolated neurons (where most DA receptors are located) are lost during the process of dissection. Third, the resting membrane potential of a cell, which determines response to agonists, is influenced by neighboring synaptically connected cells. These neighboring cells and their normal connections are not present in culture. Fourth, striatonigral neurons used in this experiment were GABAergic cells, not cholinergic cells, like the motor neurons that we have studied. As mentioned earlier, GABAergic neurons of the striatum express a complement of DA receptors different from those expressed in cholinergic cells (Surmeier *et al.* 1996). Interestingly, cholinergic neurons of the striatum coexpress primarily D5 and D2 receptor subtypes while the striatonigral cells studied by Surmeier *et al.* express primarily D1 and D3 subtypes (Surmeier *et al.* 1996). When we compare the transmembrane regions of the DOP-1 receptor (which are the regions of highest conservation between DA receptors), we find that DOP-1 is more similar to the mammalian D5 receptor than to the D1 receptor, suggesting that, even at the level of receptor expression, the mechanisms of DA signaling in cholinergic neurons may be conserved between *C. elegans* and mammals.

While we have shown that coexpressed D1- and D2-like receptors can have opposite effects on neurotransmitter release within the confines of a single cell type, we have not determined where in the cell the receptors function. DOP-1

and DOP-3 could be functioning at the same or different areas of the cell to modulate neurotransmission. For example, one or both of the receptors could act directly at the synapse to modulate vesicle fusion or they could function postsynaptically to modulate neuron excitability.

### D1- and D2-like receptors also have opposite effects on acetylcholine release in the mammalian brain

We have shown that DOP-1 and DOP-3 receptors have opposite effects on acetylcholine release, and it has long been recognized that D1- and D2-like DA receptors have opposite effects on acetylcholine release in the striatum (Lehmann and Langer 1983). Agonists for D1 receptors enhance acetylcholine release while agonists for D2 receptors reduced acetylcholine release. The cellular site of the action of D1- and D2-like receptors that modulate acetylcholine release in the striatum has not been resolved and is complicated by the intimate crosstalk between the cholinergic interneurons that release acetylcholine and the GABAergic neurons that innervate them as both neuron types express D1- and D2-like receptors. These complications do not exist in *C. elegans*, as the site of DA receptor function can be established using cell-specific promoters to express receptor transgenes back into specific neurons of receptor mutant animals. Using this approach, we found that D1- and D2-like receptors have antagonistic effects on acetylcholine release by acting directly in the cholinergic neurons themselves.

### D1-like receptors can act through Gαq, and D2-like receptors can act through Gαo in the brain

We showed that, in the cholinergic motor neurons of *C. elegans*, DOP-1 receptors act through the Gαq protein and PLCβ and DOP-3 receptors act through the Gαo protein (this work and Chase *et al.* 2004). However, the long-held model of D1 and D2 DA signaling in the mammalian brain is that D1 receptors couple to Gαs/olf to stimulate adenylyl cyclase activity and that D2 receptors antagonize this signaling by acting through Gαi/o to inhibit adenylyl cyclase activity. The opposite effects of D1 and D2 signaling on adenylyl cyclase activity suggest a simple model to explain the antagonistic effects of coexpressed DA receptor signaling; however, whether or not this antagonism occurs within a single cell remains largely untested. When tested in cell culture experiments, DOP-1 and DOP-3 receptors can increase and decrease cAMP levels, respectively (Sanyal *et al.* 2004; Sugiura *et al.* 2005), but this is not the coupling that we have identified *in vivo* despite the fact that Gαs is expressed in the cholinergic motor neurons and is thus available for coupling to the DOP-1 receptor (Korswagen *et al.* 1997).

Analogous to our *in vivo* results in *C. elegans*, most D2-like DA receptors in the striatum are coupled to Gαo, as D2-like receptors largely lost their coupling to G proteins in brains from Gαo knockout mice (Jiang *et al.* 2001). Furthermore, Gαo is regulated by RGS9-2 in medium spiny neurons (Cabrera-Vera *et al.* 2004), and we have found that the homologous protein EGL-10 regulates GOA-1/Gao coupled to DOP-3 in *C. elegans* (Figure 1A and Chase *et al.* 2004). Finally, D1-like receptors in the mammalian brain can couple to Gαq (Wang *et al.* 1995), and we have shown that the *C. elegans* DOP-1 receptor also couples to Gaq (this work and Chase *et al.* 2004). Thus, the molecular components of DA signaling are conserved between *C. elegans* and mammals. Indeed, each of the G protein-signaling molecules that we have identified in *C. elegans* is conserved in mammals and is expressed in DA-receptive neurons of the brain. If the mechanism of Gαq and Gαo signaling is conserved from *C. elegans* to mammals, signaling by D1-like receptors through Gαq and D2-like receptors through Gαo could explain the antagonistic effects observed for these receptors on acetylcholine release in mammals.

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Supporting Information
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# Coexpressed D1- and D2-Like Dopamine Receptors Antagonistically Modulate Acetylcholine Release in *Caenorhabditis elegans*

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**Files S1-S3**

**Supporting Movies**

Files S1-S3 are available for download as .avi files at

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File S1: Swimming phenotype of wild-type animals. Wild-type animals continue to swim after six minutes of vigorous activity. Shown is a 5 sec movie taken six minutes after animals were placed in water.

File S2: Swimming phenotype of *dat-1* mutants. *dat-1* mutants become paralyzed within six minutes of vigorous activity. Shown is a 5 sec movie taken six minutes after animals were placed in water.

File S3: Swimming phenotype of *dat-1; dop-3* double mutants. *dat-1; dop-3* double mutants continue to swim after six minutes of vigorous activity. Shown is a 5 sec movie taken six minutes after animals were placed in water.