Title:
Coexpressed D1- and D2-like dopamine receptors antagonistically modulate
acetylcholine release in *Caenorhabditis elegans*

Authors and author addresses: Andrew T. Allen*, Kathryn N. Maher§**, Khursheed A
Wani§**, Katherine E. Betts*, and Daniel L Chase*,†

*Department of Biochemistry and Molecular Biology and §Molecular and Cellular Biology
Program, University of Massachusetts, Amherst, Massachusetts, 01003

** these authors contributed equally to this work
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¹ Corresponding author: 710 North Pleasant St., LGRT 918, University of Massachusetts, Amherst, MA 01003. Phone: (413)-545-0631. fax: (413) 545-3291, e-mail: danchase@biochem.umass.edu
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-like and D2-like receptors are coexpressed in many neurons of the mammalian brain, it is unclear how signaling by these coexpressed receptors interact to modulate the activity of the neuron in which they are expressed. D1-like 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 (SWIP). 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-like 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.
MATERIALS AND METHODS

Nematode culture: Worm strains were maintained at 20ºC under standard conditions and double and triple mutants were generated using standard methods (Brenner 1974). Mutants analyzed in behavioral assays shown 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, ace-1(nd35) X.

DA resistance screen: Synchronized populations of fourth larval stage (L4) N2 animals were mutagenized with 30µM ethyl methanesulfonate for four hr and cultured on NGM plates for 24 hr before F1 embryos were harvested by bleach treatment of gravid adults. Synchronized L4 F1 progeny were cloned to individual wells of untreated flat-bottomed 96 well plates, each well containing 50µL of OD$_{550}$=10 OP50 culture suspended in S complete media. Worm cultures were grown at 20ºC for three days in a humidified container and were then washed three times with 100µL of water and tested for resistance to a 40mM 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 more than four 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 >75% of the progeny were resistant to 40mM liquid DA after four 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, F2 progeny of mutagenized animals were placed onto agar plates containing 40mM DA. After 20 min, rare animals were selected that were
capable of spontaneous movement. The F2 animals transferred to DA plates in this screen originated from a population of F1 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 are from a single F1 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, F1 progeny of mutagenized animals were grown individually in liquid culture microtiter plates. The F1 animals are heterozygous for induced mutations and give rise to F2 broods containing both mutant heterozygotes and homozygotes. Because the DA resistance test was performed on a population of F2 progeny from a single F1 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. Secondly, 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 F2 generation. Rehomozygosed mutants were identified by placing F2 animals on agar plates containing 40mM DA and selecting animals that were resistant to paralysis after four 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, 50ng/µL of pCL31 (acr-2::GFP) and 25ng/µL of pCL34 (acr-2::DOP-3) plasmids were co-injected with 15ng/µL of pJK4 (myo-2::GFP). For rescue of DOP-3 in the GABAergic motor neurons of *dat-1; dop-3* mutants, 50ng/µL of pCL32 (unc-47::GFP) and 25ng/µL of pCL35 (unc-47::DOP-3) were co-injected with 15ng/µL of pJK4. For rescue of DOP-3 in the cholinergic motor neurons of *dop-3 ace-1* mutants, 50ng/µL of pCL31 and 25ng/µL of pCL34 were co-injected with 15ng/µL of pJK4. For rescue of DOP-1 in the cholinergic motor neurons of *dop-3 dop-1 ace-1* mutants, 50ng/µL of pCL31 and 25ng/µL of pCL33 (acr-2::DOP-1) were co-injected with 15ng/µL of pJK4. Five independent transgenic lines were established for each experimental group, and 50 L4 animals from each line were selected that displayed the most complete expression of GFP in the cholinergic or GABAergic motor neurons 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 shown in Figure 3C an *acr-2::GFP* construct (pCL31) was coinjected with pL15EK (both at 50ng/ul) into the strain MT8189, GFP-positive animals were identified and mated with *unc-47::mCherry* expressing males (strain IZ501(Ufls34), 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 five sec observation period. Assays were repeated in triplicate for a total of at least 75 animals per strain. For acute aldicarb exposure, 1mM aldicarb plates were made by adding a 0.5M stock solution to molten low-salt agar at 55°C to a final concentration of 1mM. Plates were stored inverted in the dark at room temperature for 24 hr, then stored at 4°C and used within one week. Plates were allowed to equilibrate at room temperature for 30 min prior to the assay. Approximately twenty-five young adult animals were picked away from food and placed in the center of a 1mM aldicarb plate and prodded every five min with a platinum worm pick and scored for paralysis. For all figures that include *glr-1(nd38)*
mutants paralysis was defined as the inability to exhibit at least one body bend in a five sec period following prodding. For all other figures paralysis was defined as the inability to exhibit at least two body bends in a five 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 35µl of HB101 bacteria (A600=0.70-0.75) across each plate and incubation overnight at 37ºC. Data were collected for six animals per condition for a total of 30 measurements per condition. Percent slowing was calculated by dividing the difference between locomotion rates on and off food by the locomotion rate off food. Swimming induced paralysis assays were performed by picking 10 L4 animals away from food and then placing them in a 50µL 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 Figures 2B, 3B and 4 were done using two-tailed Students t-test. In all other figures error bars represent the means of three trials and SEM. In these other figures (figures 2A, 5A- C, and 6A, B) we compared the curves of each mutant to the wildtype or other appropriate control (see below) using a
two-way ANOVA with repeated measures followed by a Bonferromi 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, 40mM; p<0.0001) (dop-3(vs106), glr-1(nd35) and glr-1(A/T) at 15, 20,30,40, 60 and 80mM; 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(nd35) 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 they were different (at t= 20 min. p<0.001 at t=25-65 min. p<0.0001). In Figure 6B we compared the curve for 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 they were different (at t= 20 min. p<0.001 at t=25-65 min. p<0.0001).
INTRODUCTION

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; Kelley 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 they 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 C. 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 G\textsubscript{\alpha}s, G\textsubscript{\alpha}i/o,
and Gαq, are expressed throughout the *C. elegans* nervous system (Jansen et al. 1999).

*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-like and D2-like receptors act antagonistically in vivo we performed a genetic screen for DA signaling mutants in *C. elegans*. The genes we identified allowed us to show that signaling through coexpressed D1- and D2-like receptors oppositely modulate acetylcholine release by acting through Gαq and Gαo signaling pathways, respectively, directly in the cholinergic neurons themselves.
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 showed previously act antagonistically to mediate DA’s control of locomotion in *C. elegans* (Fig. 1A and Chase *et al.* 2004). The DOP-3 receptor is coupled to the G protein G\( \alpha \)/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\( \beta \)
(PLCβ/EGL-8) to generate diacylglycerol (DAG) and inositol trisphosphate (IP3) from phosphotidylinositol 4,5-bisphosphate (PIP2). Gαq/EGL-30 signaling is terminated by the RGS protein EAT-16 and Gβ5/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 (2)AVD, (2)AVA, (2)AVE, (2)AVB and (2)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 per min) than wild-type animals (2.1 ± 0.1 reversals per min) and this effect was dominant (*glr-1(nd38)/+* heterozygotes reversed 35.6 ± 0.3 times per 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 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
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 a proline change (S668P) in the third transmembrane domain (TMDIII) of GLR-1 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 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 like the GLR-1 A/T transgene, 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 only innervate 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 acetylcholine release into the neuromuscular junction 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 et al. 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 40mM 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 40mM DA more than 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). Since 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, this suggests 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 can not block excess neural activity caused by hyper-activity 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.* 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 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 now 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 (SWIP). Wild-type animals placed in water swim vigorously for more than 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 Supplemental Movies 1-3).

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 more than 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 the DOP-3 receptor in cholinergic motor neurons, we expressed the DOP-3 receptor from the acr-2 promoter, which is active in 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
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 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 4A, 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, 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), dgk-1(sy428)* null mutants, and *egl-30(tg26)* gain of function mutants (refer to 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 alpha subunit) or that 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 should 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.

**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).

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. To test this we 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 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 5B, 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 (for example,
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 to the acr-2::DOP-3 transgene to reduce the aldicarb hypersensitivity of 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 themselves 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 now 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 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 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 mutation in dop-1 and dop-3 have opposite effects on acetylcholine release.
In other experiments Surmeier and coworkers (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 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 only expressed 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 we have studied. As mentioned earlier, GABAergic neurons of the striatum express a different complement of DA receptors than 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 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 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-like 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-like 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 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 \( \text{G}^{\alpha q} \) and D2-like receptors can act through \( \text{G}^{\alpha o} \) in the brain: We showed that in the cholinergic motor neurons of *C. elegans*, DOP-1 receptors act through the \( \text{G}^{\alpha q} \) protein and PLC\( \beta \) and DOP-3 receptors act through the \( \text{G}^{\alpha o} \) 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 \( \text{G}^{\alpha s/olf} \) to stimulate adenylyl cyclase activity and that D2 receptors antagonize this signaling by acting through \( \text{G}^{\alpha i/o} \) to inhibit adenylyl cyclase activity. The opposite effects of D1 and D2 signaling on adenylyl cyclase activity suggests 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 we have identified *in vivo* despite the fact that \( \text{G}^{\alpha s} \) 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 \( \text{G}^{\alpha o} \), as D2-like receptors largely lost their coupling to G proteins in brains from \( \text{G}^{\alpha o} \) knockout mice (Jiang *et al.* 2001). Further, \( \text{G}^{\alpha o} \) 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 \( \text{G}^{\alpha q} \) (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 we have identified in *C. elegans* is conserved in mammals and is expressed in DA-receptive neurons of the brain. If the mechanism of G\(_\alpha\)q and G\(_\alpha\)o signaling is conserved from *C. elegans* to mammals, signaling by D1-like receptors through G\(_\alpha\)q and D2-like receptors through G\(_\alpha\)o could explain the antagonistic effects observed for these receptors on acetylcholine release in mammals.
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FIGURE LEGENDS

FIGURE 1. Mutations and signaling pathways that control locomotion in C. elegans. (A), Schematic representation 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 gray ovals. Names of C. elegans signaling proteins are shown inside the ovals and names of their mammalian homologs are shown outside 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. Black boxes indicate identical residues, gray boxes indicate 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.

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 ± standard error of the mean (s.e.m.) 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 (white bars) and presence of bacteria (black 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 (Students t-test, *P<0.01, **P<0.0001). The percent slowing in the presence of bacteria for each strain is shown at the right.
FIGURE 3. Swimming induced paralysis is mediated by DOP-3 signaling in the ventral cord motor neurons. Quantitative analysis of swimming induced paralysis. (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 (right bracket). A few other neurons located in the head (left bracket) express GFP or mCherry but these neurons do not innervate body wall muscles to control locomotion. Scale bar 20µm. (B), SWIP behavior of control, non-transgenic 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. Light gray bars represent measurements from control strains carrying empty vector transgenes, which have promoters but no receptor sequences. Black 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. Asterisk indicates that receptor expression gave significant rescue compared to the control (unpaired Students 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 GABAergic motor neurons.

FIGURE 4. Quantitative analysis of SWIP behavior of dopamine signaling mutants. (A-D), Each measurement shown 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 significant difference from dat-1 mutants (Students t test, **p<0.0001).
Asterisks in B indicate significant difference between dat-1; dop-1 dop-3 triple mutant and double mutants indicated (Students 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 1mM aldicarb. Each data point represents the mean ± standard error of the mean (s.e.m.) for three trials totaling at least 75 animals.

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 aldicarb. Each data point for nontransgenic animals represents the mean ± standard error of the mean (s.e.m.) 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, 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.