2009

Dopamine Controls Locomotion by Modulating the Activity of the Cholinergic Motor Neurons in C. elegans

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DOPAMINE CONTROLS LOCOMOTION BY MODULATING THE ACTIVITY OF THE CHOLINERGIC MOTOR NEURONS IN *C. ELEGANS*

A Thesis Presented

by

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Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

May 2009

Biochemistry and Molecular Biology

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DEDICATION

To Donnie Johnson: you'd better be smiling up there.

ACKNOWLEDGMENTS

I would like to thank Kathryn Maher, Khursheed Wani, and Katherine Betts for advice, assistance, and discussion; scientific or otherwise. Also, I would like to thank my family for their love, support, and confidence. Finally, I would like to thank Daniel Chase for instilling in me a passion for meticulous and exacting science: better late than never.

ABSTRACT

DOPAMINE CONTROLS LOCOMOTION BY MODULATING THE ACTIVITY OF THE CHOLINERGIC MOTOR NEURONS IN *C. ELEGANS*

MAY 2009

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Dopamine is an important neurotransmitter in the brain, where it plays a regulatory role in the coordination of movement and cognition by acting through two classes of G protein-coupled receptors to modulate synaptic activity. In addition, it has been shown these two receptor classes can exhibit synergistic or antagonistic effects on neurotransmission. However, while the pharmacology of the mammalian dopamine receptors have been characterized in some detail, less is known about the molecular pathways that act downstream of the receptors. As in mammals, the soil nematode *Caenorhabditis elegans* uses two classes of dopamine receptors to control neural activity and thus can serve as a genetic tool to identify the molecular mechanisms through which dopamine receptors exert their effects on neurotransmission. To identify novel components of mammalian dopamine signaling pathways, we conducted a genetic screen for *C. elegans* mutants defective in exogenous dopamine response. We screened 31,000 mutagenized haploid genomes and recovered seven mutants. Five of these mutants were in previously-identified dopamine signaling genes, including those encoding the Gα proteins GOA-1 (ortholog of human $G\alpha_o$) and EGL-30 (ortholog of human $G\alpha_q$), the diacylglycerol kinase DGK-1 (ortholog of human DGKθ), and the dopamine receptor

DOP-3 (ortholog of human D2-like receptor). In addition to these known components, we identified mutations in the glutamate-gated cation channel subunit GLR-1 (ortholog of human AMPA receptor subunits) and the class A acetylcholinesterase ACE-1 (ortholog of human acetylcholinesterase). Behavioral analysis of these mutants demonstrates that dopamine signaling controls acetylcholine release by modulating the excitability of the cholinergic motor neurons in *C. elegans* through two antagonistic dopamine receptor signaling pathways, and that this antagonism occurs within a single cell. In addition, a mutation in the putative Rab GTPase activating protein TBC-4 was identified, which may suggest a role for this Rab GAP in synaptic vesicle trafficking. Subsequent behavioral and genetic analyses of mutants in synaptic vesicular trafficking components implicate RAB-3-mediated vesicular trafficking in DOP-3 receptor signaling. These results together suggest a possible mechanism for the regulation of dopamine receptor signaling by vesicular trafficking components in the cholinergic motor neurons of *C. elegans*.

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CHAPTER I

INTRODUCTION

Dopamine signaling in mammals

Dopamine is a neurotransmitter whose importance in regulating neural activity in the mammalian brain is well established. In mammals, dopamine binds to five seven transmembrane heterotrimeric G-protein coupled receptors to exert its effects on neurotransmission. These five mammalian dopamine receptors have been grouped into two classes based on biochemical and pharmacological criteria: D1-like and D2-like.

Previously, it has been shown in mammals that D1-like and D2-like receptors can have both antagonistic and synergistic effects on neurotransmission (Missale *et al.* 1998). D1-like receptors increase the excitability of the neuron by stimulating cyclic AMP-dependent processes or by increasing the availability of intracellular calcium, whereas D2-like receptors attenuate the excitability of the neuron by counteracting these processes (Neve *et al*. 2004). Additionally, D1-like and D2-like receptors can form hetero-oligomers through which unique physiological responses can be elicited (Lee *et al.* 2004). While the downstream effects of these receptors on neural activity have been heavily investigated both *in vivo* and *in vitro*, the molecular mechanisms through which dopamine receptor signaling influences neurotransmission remain largely undiscovered, in part due to the lack of a genetically tractable model organism with which to answer these questions.

Dopamine signaling in *C. elegans*

The soil nematode *C. elegans* is an excellent tool for the genetic analysis of neurotransmission with a simple nervous system consisting of 302 neurons (White *et al.* 1986). Eight of these neurons synthesize dopamine (Sulston *et al.* 1975), and it has been shown that in response to mechanical stimulation these neurons release dopamine which functions extrasynaptically to control locomotion (Sawin *et al.* 2000; Chase *et al.* 2004). Importantly, many components of G-protein coupled receptor signaling in the mammalian brain have homologs in *C. elegans*, suggesting that the molecular mechanisms mediating dopamine signaling in the worm may be conserved in mammals. Among these conserved components are the dopamine receptors DOP-1 and DOP-3, which are homologous to the D1 and D2-like receptors present in the mammalian brain, respectively (Suo *et al.* 2002; Sugiura *et al.* 2005). These two receptors are co-expressed in the cholinergic motor neurons of *C. elegans*, where DOP-3 signals through the $G\alpha_o$ homolog GOA-1 to inhibit locomotion, and DOP-1 antagonizes DOP-3 signaling by positively regulating locomotion through the $G\alpha_q$ homolog EGL-30 (Lackner *et al.* 1999; Chase *et. al*. 2004). These observations firmly establish the cholinergic motor neuron of *C. elegans* as a model system to investigate the antagonistic mechanism of dopamine signaling.

Exposure of *C. elegans* to high levels of exogenous dopamine causes paralysis by acting through the DOP-3 receptor, and it has been shown that mutants in the molecular components responsible for inhibiting locomotion in response to dopamine are resistant to these paralytic effects (Chase *et al.* 2004). Therefore, forward genetic approaches can be used to isolate additional components of dopamine signaling pathways in *C. elegans*

using exogenous dopamine resistance as a selective criteria. Such screens have been done previously and have succeeded in identifying several components of dopamine signaling in *C. elegans* conserved in mammals (Chase *et al.* 2004). However, *dop-3* mutants were not isolated in these screens, and given the importance of the DOP-3 receptor in the exogenous dopamine response, we reasoned that there may be additional components of dopamine signaling that remain unidentified. Therefore, we conducted a modified genetic screen to isolate additional components of the exogenous dopamine response. As a result of our screen, we have established that dopamine controls the release of acetylcholine by modulating the excitability of the cholinergic motor neurons through two antagonistic dopamine receptor signaling pathways, and that this antagonism occurs within a single cell.

Rab GTPases and their putative role in dopamine signaling

Rab GTPases are important molecular switches that regulate the transport, docking, and fusion of vesicles in many cellular processes (Fukuda 2008), and Rab activity can be modified by several additional proteins. Guanine nucleotide exchange factors (GEFs) enhance a Rab protein's association with vesicular membranes, and Rab GTPase activating proteins (Rab GAPs) enhance the intrinsic GTPase activity of the Rab protein, resulting in the dissociation of the Rab protein from the membrane to which it is bound.

Two Rab GTPases in *C. elegans* have been localized to the pre-synaptic terminals of cholinergic motor neurons: RAB-3 (homolog of mammalian Rab3A) and AEX-6 (homolog of mammalian Rab27A). Both RAB-3 and AEX-6 have been shown to play important roles in synaptic transmission in *C. elegans* (Nonet *et al.* 1997; Mahoney *et al.*

2006), and Rab3A has been shown to work in a similar capacity in mammals (Geppert *et al.* 1997, Leenders *et al.* 2001, Wang *et al.* 2008). However, the subtle behavioral defects of *rab-3* mutants combined with an incomplete mechanistic understanding of AEX-6-mediated vesicular docking raise interesting questions as to exactly how RAB-3 and AEX-6 facilitate neurotransmission.

As a result of our genetic screen for mutants resistant to exogenous dopamine, we identified a mutant in the *tbc-4* gene, which is predicted to encode a Rab GAP in *C. elegans*. All known Rab GAPs contain a highly conserved TBC domain which is essential for Rab GAP function, and the TBC-4 protein is predicted to contain a single TBC domain; therefore, it is likely that the *tbc-4* gene encodes a Rab GAP in *C. elegans*. In addition, the resistance of a *tbc-4* mutant to exogenous dopamine suggests that the regulation of Rab GTPase activity may be influenced by dopamine signaling, and this regulation may provide a mechanism through which dopamine controls acetylcholine release.

CHAPTER II

MATERIALS AND METHODS

Worm genetics

Strains

All worm strains were maintained at 20º under standard conditions (unless otherwise noted), and all double and triple mutants were generated using standard methods (Brenner 1974). The strains used in this study were: N2 Bristol, *ace-2(g72) I, goa-1(sa734) I, egl-30(tg26) I, eat-16(ad702) I, aex-6(sa24) I, cat-2(e1112) II, rab-3(js49 II), glr-1(nd38) III, rbf-1(js232) III, dat-1(ok157) III, dop-2(vs105) V, dgk-1(sy428) X, aex-3(js814) X, dop-3(vs106) X, dop-1(vs100) X, ace-1(nd35) X*, *tbc-4(ok3041) X.*

Isolation of dopamine resistant mutants

N2 animals were mutagenized with ethyl methanesulfonate and cultured on NGM plates. Staged L4 F1 progeny were cloned to individual wells of untreated flat-bottomed 96 well plates, each well containing 50 μL of OD_{550}=10 OP50 culture suspended in S complete media. Plates were grown at 20 C for three days in a plastic container lined with moistened paper towels. Cultures were then washed three times in water and tested for resistance to 120 μL of a 40 mM liquid dopamine solution. Mutants were isolated as resistant if 25 percent of a well remained thrashing in dopamine solution for more than four minutes. Primary isolates were immediately rescued, and several clones were inoculated into liquid culture to homozygose the mutation. Clones whose progeny were

greater than 75 percent resistant to 40 mM liquid dopamine were deemed dopamine resistant and the mutants were subsequently mapped and outcrossed on 40 mM dopamine plates.

SNP mapping of screen isolates

Isolates were mapped to a chromosome by mating to the polymorphic mapping strain CB4856, identifying cross-progeny, and rehomozygosing the dopamine resistance of the mutation in the F2 generation. Since the mutation was homozygosed in the N2 Bristol background, PCR and digestion analysis of a panel of single nucleotide polymorphisms (SNPs) located at the center of each chromosome was used to identify the chromosome on which the mutation is present based on recombination frequency (Wicks *et al.* 2001). This same strategy was applied to fine-map the location of the mutations to specific regions of a chromosome using a panel of chromosome-specific SNPs.

Genetic complementation

For complementation analysis of screen isolates, mutants in candidate genes were crossed into PD4788 males expressing *myo-2::*GFP (Edgely *et al.* 1999), and F1 heterozygote males were selected based on fluorescence of the pharyngeal muscle. These animals were then crossed into prospective isolates from the screen, and heterozygotes from this cross were then tested for dopamine resistance.

Two-factor positional mapping

For two-factor mapping of the *ace-1(nd35)* mutation, the *dyn-1(ky51) X* and *sup-10(n983) X* mutations were used as flanking visible markers. *dyn-1* animals are un-coordinated (Unc) at 25º, and *sup-10* mutants are long, egg-laying defective, and un-coordinated (Sup) (Clark *et al.* 1997; Greenwald and Horvitz 1985). As *sup-10(n983)* masks the phenotype of *dyn-1(ky51)* animals, mapping recombinants could only be selected that were Dyn-non-Sup. The recombinant chromosome was homozygozed through self-fertilization and selection of Dyn-never-Sup populations, and the resultant populations were assayed for dopamine resistance.

Generation and analysis of transgenic animals

Tissue-specific rescue of DOP-3 and DOP-1

For tissue-specific 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*)* were co-injected with 15 ng/µL of pJK4 (*myo-2::*GFP). For tissue-specific 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 (*myo-2::*GFP). For tissue-specific rescue of DOP-3 in the cholinergic and GABAergic neurons of *dat-1; dop-3* mutants, 50 ng/µL of pCL31 and pCL32 were co-injected with 25 ng/µL of pCL34 and pCL35. For tissue-specific 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 tissue-specific 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 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 sub-cloning procedures. *acr-2* is a cholinergic neuron specific promoter (Hallam *et al.* 2000), and *unc-47* is a GABAergic neuron specific promoter (Eastman *et al.* 1999).

Rescue of dopamine sensitivity in *tbc-4* mutants using a full-length TBC-4 PCR product

A 12.5 kb fragment of genomic DNA containing the complete coding sequence of TBC-4, 5 kb of 5' upstream regulatory sequence, and 1 kb of 3' UTR was amplified using the Qiagen LongRange PCR kit (Qiagen). 50 ng/μL of PCR product was co-injected with 25 ng/μL of pJK4 into *tbc-4(ok3041)* animals. Five independent transgenic lines were established based on pharyngeal GFP florescence, and 50 young adult animals from each line were selected and assayed for the exogenous dopamine resistance. Five control lines carrying only pJK4 were generated and assayed in parallel.

Determination of the expression pattern of *tbc-4*

A 12 kb fragment of genomic DNA containing 9 kb of sequence upstream from the predicted start codon of TBC-4 and the entire 3 kb of the first intron was fused to GFP amplified from pPD49.77 (Addgene) using PCR fusion (Hobert 2002). 50 ng/μL of the PCR product was co-injected with 50 ng/μL of pL15EK into *lin-15(n765ts)* animals, and three independent transgenic lines were established based on the rescue of the multiple vulva phenotype (Muv) of *lin-15* animals at 20º.

For localization of *tbc-4* expression to the cholinergic motor neurons, the *dop-1::RFP* integrated line LX797 was crossed into *tbc-4::*GFP males and cross-progeny were identified by florescence microscopy.

For co-localization of TBC-4 with RAB-3, transgenic animals expressing *rab-3::*mCherry were created by injecting 50 ng/μL of pJORG1901 (*rab-3::mCherry*) (a kind gift of Erik Jorgensen) with 50 ng/μL of pL15EK into *lin-15(n765ts)* animals and isolating transgenic lines by the rescue of the Muv phenotype. Then, *rab-3::*mCherry animals were crossed into *tbc-4::*GFP males and cross-progeny were identified by florescent microscopy.

All images were taken with a Cool Snap EZ camera on a Nikon Eclipse FN1 microscope equipped with epiflouresence using a 60x oil immersion objective. All imaged animals were immobilized with 2mM levamisole and mounted on microscope slides containing 2% agarose. Merged images were created using Nikon Elements imaging software.

Behavioral analysis of mutants

Dopamine response

10 young adults for each strain to be assayed were incubated undisturbed for 4 minutes on plates containing 40 mM dopamine, and then scored for paralysis. Animals were considered paralyzed when they were not able to exhibit at least one spontaneous body bend in a 5 sec period. A body bend was defined as the movement of the posterior bulb of the pharynx through either a minimum of maximum amplitude. Assays were repeated five times for a total of 50 animals per strain. Dopamine plates were prepared as previously described (Chase *et al.* 2004).

Dopamine dose-response

25 young adults for each strain to be assayed were incubated undisturbed for 10 minutes on plates containing the indicated concentration of dopamine, and then scored for paralysis. Animals were considered paralyzed when they were not able to exhibit at least one spontaneous body bend in a 5 sec period. Assays were repeated in duplicate for a total of 50 animals per strain.

Chronic aldicarb exposure

Chronic aldicarb sensitivity assays were done as previously described (Miller *et al.* 1999). Briefly, enough L4 animals of each strain were cloned to aldicarb plates of the indicated concentration to spawn 300 progeny on a drug-free NGM plate and allowed to grow at 20º for 96 hours. The resultant progeny were counted and data was plotted as the

percent population size as compared to the no drug control. Each condition was tested in triplicate for all strains.

Acute aldicarb exposure

1 mM aldicarb plates were made by adding a 0.5 M stock solution to molten low-salt agar at 55º to a final concentration of 1 mM. Plates were stored inverted in the dark at room temperature for 24 hours, then stored at 4º and used within one week. Plates were allowed to equilibrate at room temperature for 30 minutes prior to the assay. 25 adult animals were picked away from food and placed in the center of a 1mM aldicarb plate and prodded sharply every five minutes with a platinum worm pick and scored for paralysis. Paralysis was defined as the inability to exhibit at least one body bend in a five second period following prodding. Each assay was done in duplicate for a total of 50 animals per strain.

Basal slowing

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 5 consecutive 20-second intervals in the presence and the absence of HB101 bacteria. Data was collected for 6 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

10 L4 animals were picked away from food and placed in a 50 uL water droplet on a Menzel Glaser 10-well diagnostic slide #X1XER308B# and scored for movement after 10 minutes. Movement was scored as the presence of the alternating C-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 (as defined above). This assay was repeated for a total of 50 animals per strain.

Egg laying

Egg laying assays were done as previously described (Chase and Koelle 2004). Briefly, 30 staged young adult animals for each strain were dissolved in 20 % commercial bleach and the eggs held *in utero* were counted for each animal within 5 minutes.

Sequence analysis of Rab GAPs

Domain comparison of Rab GAPs

Predicted protein sequences of selected Rab GAPs were obtained from the NCBI protein database (http://www.ncbi.nlm.nih.gov/) and the predicted domains contained within the coding sequence were determined using Protein SMART (http://smart.embl-heidelberg.de/). Proteins were graphically aligned to illustrate the location of the TBC-4 domain in all selected sequences.

Sequence alignment of Rab GAPs

Protein sequences of selected Rab GAPs were aligned using ClustalX multiple sequence alignment software (http://www.clustal.org/). The ALN file was then processed using the BoxShade online server (http://www.ch.embnet.org/software/BOX_form.html) to visually represent conserved amino acid residues (v3.2.1).

CHAPTER III

RESULTS

Screen isolates suggest that dopamine controls acetylcholine release by modulating the activity of the cholinergic motor neurons

We completed a clonal liquid screen of 31,241 mutagenized haploid genomes for mutants resistant to the paralytic effects of exogenous liquid dopamine (Fig. 3.1). We identified seven mutants from the screen (Table 3.1) that were subsequently mapped and outcrossed four times using 40 mM dopamine plates as the selective criteria, as it proved difficult to re-homozygose mutants using the liquid dopamine assay. From the screen, we identified four previously identified components of dopamine signaling in *C. elegans*, including the D2-like receptor DOP-3. These results confirm that our genetic screening method is an effective tool to isolate mutants in endogenous dopamine signaling components.

In addition, this screen could be continued as it is not fully saturated. It is estimated that in approximately every 2,000 ethylmethanesulfonate mutagenized *C. elegans* haploid genomes, a mutation is caused in each gene in the genome (Anderson 1995). Despite the fact that we screened enough mutagenized haploid genomes to achieve five-fold coverage of the entire genome, we failed to isolate two previously identified components of dopamine signaling in *C. elegans,* the Gβ5 subunit GPB-2 and the RGS protein EAT-16 (Chase *et al.* 2004). However, we did isolate multiple alleles of DGK-1 and a gain-of-function allele in the $G\alpha_q$ homolog EGL-30.

These results indicate that although the screen was approaching saturation, increasing the amount of genomes screened could potentially identify additional components required for dopamine signaling.

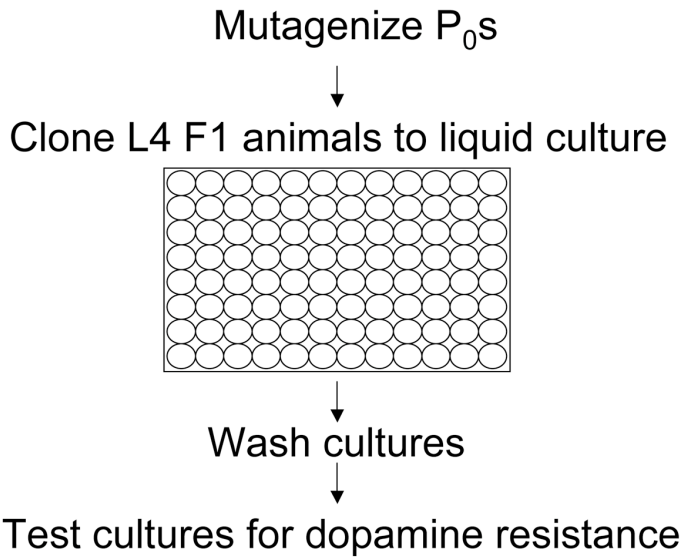


Figure 3.1. Design of a clonal liquid screen to identify mutants resistant to exogenous dopamine. F1 progeny of mutagenized P0 animals were cloned to individual wells of a 96 well plate and cultured in liquid until F2 populations reached adulthood. These cultures were then washed to remove culture media and tested for dopamine resistance. Plates were screened for wells in which one quarter of the population was dopamine resistant. Each well was founded by a single F1 hermaphrodite heterozygous for mutations in potential genes of interest, and one quarter of the F2 progeny of that animal will therefore be both homozygous for the mutation(s) and dopamine resistant.

Gene	Alleles	Mutations	Protein	Human ortholog
goa-1	*nd64*	IVS7+1G>A	G-protein alpha subunit	$G\alpha_o$
dgk-1	*nd48, nd55*	Q520X, G721E	Diacylglycerol kinase	DGKθ
egl-30	*nd50*	D151N	G-protein alpha subunit	$G\alpha_q$
dop-3	*nd41*	IVS2-1G>A	D2-like 7TM receptor	D2 7TM receptor
glr-1	*nd38*	S668T	AMPA-type glutamate-gated cation channel subunit	GluR2
ace-1	*nd35*	Q84X	Acetylcholinesterase	Acetylcholinesterase

Table 3.1. Genes and mutations identified in the dopamine resistance screen. Seven alleles of six genes were identified from a forward genetic screen of over 31,000 mutagenized haploid genomes. Four genes identified were in previously identified

components of dopamine signaling (Chase *et al.* 2004), and all genes identified are conserved in the mammalian brain. All mutations were identified via direct sequencing of purified PCR amplicons (GeneWiz, https://clims3.genewiz.com/default.aspx). Closest human orthologs were identified by protein sequence comparison using ClustalX.

Our screen also identified two mutants in components not previously implicated in dopamine signaling. We identified a gain-of-function mutant in the glutamate-gated cation channel subunit GLR-1 and a loss-of-function mutant in the class A acetylcholinesterase ACE-1.

GLR-1 is homologous to the mammalian AMPA receptor subunits in the brain (56% identity to RatGluR1) that collectively play an important role in fast excitatory neurotransmission and long-term memory (Jonas 1993; Manilow and Malenka 2002). GLR-1 is expressed in the five pairs of command interneurons which control the direction of locomotion (Chalfie *et al.* 1985; Brockie *et al.* 2001), and it is known that these neurons synapse onto the cholinergic motor neurons of the ventral nerve cord which directly control body wall muscle contraction through the release of the neurotransmitter acetylcholine (White *et al.* 1986; Richmond and Jorgensen 1999). The *glr-1(nd38)* mutation identified in the screen causes a semi-dominant hyper-reversal phenotype, and we proposed that it causes the GLR-1 channel to remain constitutively open, leading to abnormal over-stimulation of the cholinergic motor neurons and thus dopamine resistance. This hypothesis is supported by previous work in which transgenic worms carrying a constitutively open copy of GLR-1 exhibits a hyper-reversal phenotype (Zheng *et al.* 1999). The mutation present in the transgenic line was originally identified in *Mus musculus* as a spontaneous mutant with a staggering gait, and was named the Lurcher mutation (Phillips 1960). The Lurcher mutation was subsequently identified as an

alanine-to-threonine change in a highly conserved third transmembrane region of the δ2 subtype of the *M. musculus* glutamate-gated cation channel which results in a constitutively open channel (Zuo *et al.* 1997). The *glr-1* mutant identified in our genetic screen phenocopies the hyper reversal phenotype of the transgenic GLR-1(A/T) worms, and sequencing analysis demonstrates that the *glr-1(nd38)* mutation is in the same third transmembrane domain of GLR-1 as the Lurcher mutation (Fig. 3.2*A*). Therefore, we can conclude that *glr-1(nd38)* mutants are resistant to exogenous dopamine due to an increase in the excitability of the cholinergic motor neurons caused by over-stimulation from the command interneurons, most likely due to the presence of a constitutively open channel.

ACE-1 is homologous to mammalian acetylcholinesterase, which degrades acetylcholine in both synaptic terminals and neuromuscular junctions. ACE-1 is expressed in the body wall muscles of *C. elegans* and has been shown to function redundantly with ACE-2 to facilitate a wild-type rate of acetylcholine turnover in the synaptic cleft (Culotti *et al.* 1981; Culetto *et al.* 1999). The *ace-1(nd35)* mutation identified in the screen was a nonsense mutation early in the ACE-1 coding sequence and is presumed to be a null allele based on the mutation's proximity to the *ace-1(p1000)* mutation (Fig. 3.2*B*), whose protein product has been shown to have no acetylcholinesterase activity *in vitro* (Johnson *et al.* 1981). As *ace-1(nd35)* mutants are resistant to exogenous dopamine, it is likely that dopamine is controlling acetylcholine release, as a loss-of-function mutation in *ace-1* would presumably increase the amount of acetylcholine in the synaptic cleft and cause over-stimulation of the innervated muscle, resulting in resistance to exogenous dopamine.

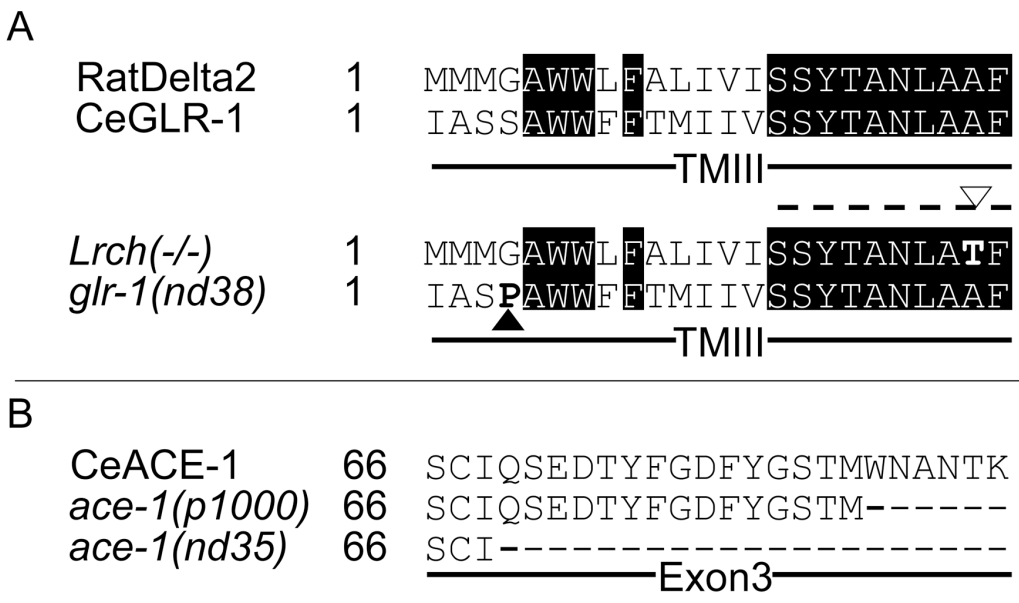


Figure 3.2. Screen isolates contain mutations in conserved domains of proteins required for neurotransmission. A.) Top: Sequence alignment of the third transmembrane (TMIII) domains of *Rattus norvigicus* δ2 and *C. elegans* GLR-1 glutamate-gated cation channel subunits displays a high degree of conservation between the two proteins. Bottom: Sequence comparison of Lurcher mutant (*Lrch(-/-)*) δ2 and *glr-1(nd38)* mutant GLR-1 demonstrates that both mutations are in residues contained in TMIII. Both *Lrch(-/-)* and *glr-1(nd38)* alleles are missense mutations that alter the polarity of the affected amino acid and presumably affect the insertion of TMIII into the membrane of the presynaptic terminal resulting in a leaky channel, a hypothesis confirmed by both physiological and behavioral analysis of the *Lrch(-/-)* mutation (Zuo *et al.* 1997; Zheng *et al.* 1999). Black boxes indicate identical amino acid residues. Open arrowhead indicates the location of the *Lrch(-/-)* mutation in δ2. Closed arrowhead indicates the location of the *glr-1(nd38)* mutation in GLR-1. Dashed line indicates the SSYTANLAAF motif shared amongst most glutamate-gated cation channel subunits. Numbering is from the first amino acid of TMIII. B.) Sequence alignment of wild-type ACE-1 with *ace-1(p1000)* and *ace-1(nd35)* mutant ACE-1 suggests that both mutant genes are likely to encode null alleles of ACE-1. ACE-1 protein encoded by *ace-1 (p1000)* is known to be truncated at W84 and has been shown to have 0% class A acetylcholinesterase activity *in vitro* (Johnson *et al.* 1981). Sequencing of *ace-1(nd35)* reveals a similar nonsense mutation in approximately the same location in the *ace-1* gene, consistent with the hypothesis that both mutants encode non-functional ACE-1. Dashes indicate amino acids not encoded in the mutant alleles of *ace-1*. Bold dashes indicate the site of the p1000 and nd35 nonsense mutations. Numbering begins at S66.

The isolation of both a gain-of-function mutation in GLR-1 and a loss-of-function mutation in ACE-1 in a screen for mutants resistant to exogenous dopamine supports the

model that dopamine signaling controls locomotion by modulating the excitability of the cholinergic motor neurons and thus acetylcholine release.

Behavioral analyses of *ace-1* and *glr-1* mutants support the model that dopamine is controlling acetylcholine release

To confirm our hypothesis that dopamine is modulating acetylcholine release in the worm, we conducted a behavioral analysis of the *ace-1* and *glr-1* mutants recovered from the screen. We chose to examine several dopamine-dependent behaviors to evaluate the role of dopamine signaling in acetylcholine release.

We first chose to characterize the *ace-1* and *glr-1* mutations by observing the resistance of the mutants to exogenous dopamine over time (Figure 3.3). *ace-1* mutants are weakly resistant to exogenous dopamine and, in fact, are less resistant than *dop-3* mutants, suggesting that ACE-1 is acting downstream of the DOP-3 receptor, a conclusion supported by the expression of ACE-1 in body wall muscle post-synaptic to the cholinergic motor neurons (Culetto *et al.* 1999). *glr-1* mutants, however, exhibit a resistance to exogenous dopamine that more closely resembles that of *goa-1* loss-of-function mutants. This suggests that GLR-1 is stimulating the cholinergic motor neurons upstream of the DOP-3 receptor, a conclusion that is again supported by the expression of GLR-1 exclusively in the command interneurons presynaptic to the cholinergic motor neurons that express DOP-3 (Brockie *et al.* 2001). Together, these results demonstrate that resistance to exogenous dopamine is conferred by mutations in genes that either increase the level of or longevity of acetylcholine release from the cholinergic motor

neurons (*glr-1, ace-1*) or decrease the inhibition of acetylcholine release from these neurons (*dop-3)*.

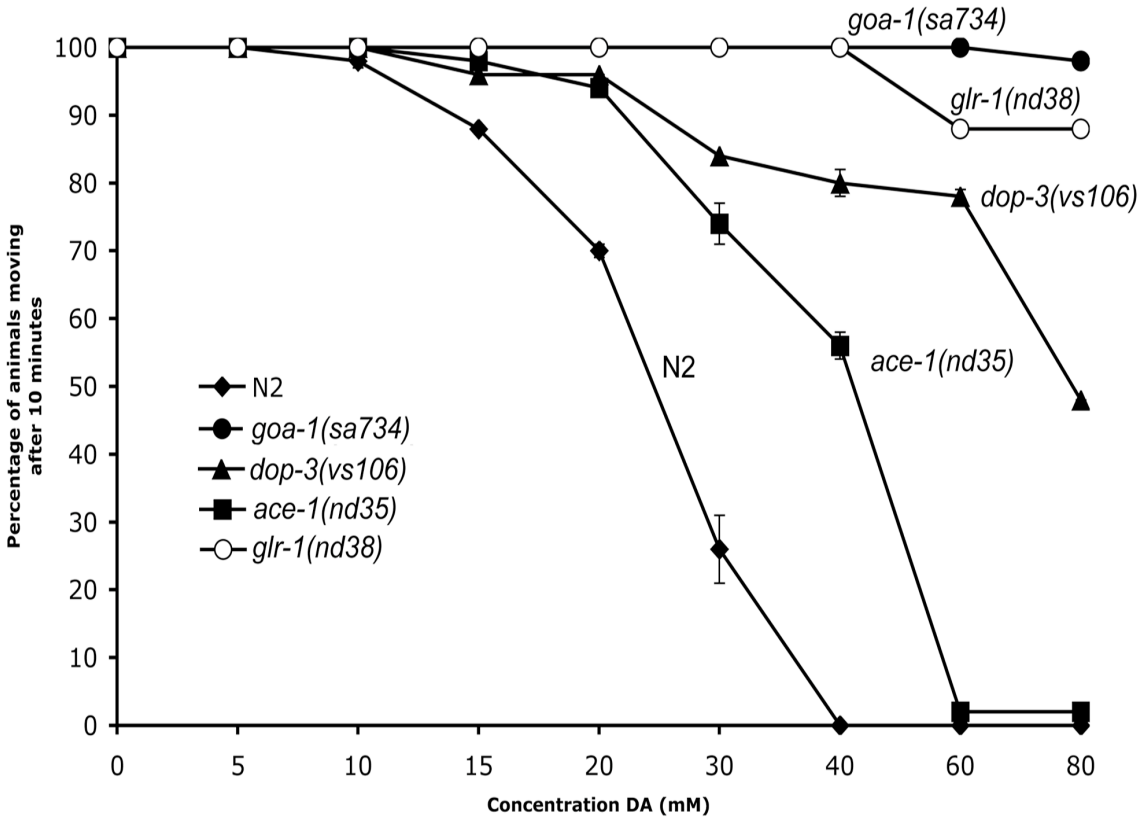


Figure 3.3. *ace-1* and *glr-1* mutants are resistant to exogenous dopamine. Dopamine resistance is a well-established paradigm for assessing the role of various molecular components in dopamine signaling (Chase *et al.* 2004). Both *ace-1(nd35)* and *glr-1(nd38)* mutants are resistant to exogenous dopamine, demonstrating that both ACE-1 and GLR-1 are required for the wild-type response to exogenous dopamine. The strong resistance of *glr-1(nd38)* demonstrates that GLR-1 modulates cholinergic motor neuron excitability upstream of DOP-3, whereas the weaker resistance of *ace-1(nd35)* demonstrates that ACE-1 is regulating cholinergic activity downstream of DOP-3. Together, these results suggest that signaling through DOP-3 modulates acetylcholine release by decreasing the excitability of the cholinergic motor neurons. Young adult animals were picked away from food and onto plates of the indicated dopamine concentration and assayed for the ability to make spontaneous body bends after 10 minutes of exposure. n=25 animals per strain. Experiments were done in duplicate for a total of 50 animals per strain. Error bars represent the standard error of the mean.

In *C. elegans*, there are several behaviors that are known to require dopamine signaling for their proper execution. More importantly, a subset of these behaviors have been shown to require signaling through the DOP-3 receptor to control locomotion. It has previously been shown that wild-type animals reduce their rate of locomotion in response to food, and that this basal slowing response is dependent on dopamine signaling through the DOP-3 receptor (Sawin *et al.* 2000; Chase *et al.* 2004). Therefore, we reasoned that if dopamine signaling was controlling acetylcholine release, both *ace-1* and *glr-1* mutants should be defective in the basal slowing response. Our experimental results confirm that both *ace-1* and *glr-1* mutants are defective in the basal slowing response, and subsequent genetic analysis of *ace-2(g72)* and *ace-2; ace-1* mutants reveals that dopamine signaling through the DOP-3 receptor reduces acetylcholine release to facilitate the basal slowing response (Fig. 3.4).

These results together demonstrate that signaling through the DOP-3 receptor conditionally regulates acetylcholine release, and that this regulation likely occurs in the cholinergic motor neurons. However, as the precise mechanisms governing both exogenous dopamine response and the basal slowing response are not entirely clear, we decided to establish a more robust paradigm to examine dopamine signaling specifically in the cholinergic motor neurons

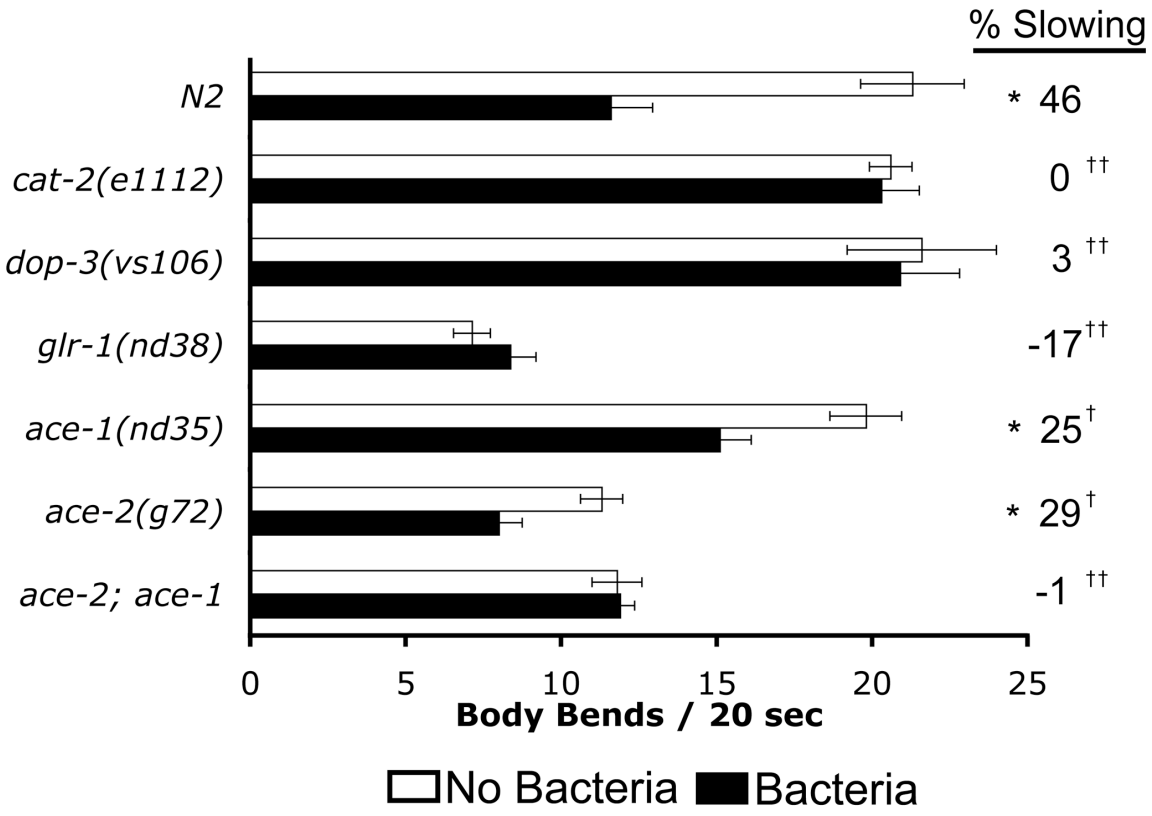


Figure 3.4. Signaling through the DOP-3 receptor decreases acetylcholine release in response to food. The basal slowing response has been shown to require signaling through the DOP-3 pathway, and mutants defective in this response are likely to harbor mutations in genes important for dopamine signaling. Both *glr-1(nd38)* and *ace-1(nd35)* are defective in the basal slowing response. *ace-1(nd35)* and *ace-2(g72)* both exhibit a partial defect in slowing, which suggests redundant function of ACE-1 and ACE-2. The *ace-2; ace-1* double mutant, however, exhibits a complete defect in basal slowing like *dop-3*, which demonstrates that signaling through the DOP-3 receptor decreases acetylcholine release in response to food to facilitate the basal slowing response. L4 hermaphrodites were staged 14-19 hrs prior to the assay at 20º. Body bends were counted for five 20-second intervals for 6 young adult worms per strain for a total of 30 independent measurements per condition. White bars indicate locomotion rate in the absence of food, and black bars indicate locomotion rate in the presence of food. Error bars represent the 95% confidence interval. Asterisks indicate slowing based on a Student's t test ($p<0.005$) and daggers indicate a defect in basal slowing as compared to the wild-type († = $p<0.025$; † † = $p<0.0005$).

SWIP is a behavior that is controlled by DOP-3 receptor signaling in the cholinergic motor neurons

Previously, it has been shown that mutants in the conserved dopamine transporter DAT-1 become paralyzed when forced to engage in vigorous motor activity, a behavioral defect referred to as swimming induced paralysis (SWIP) (McDonald *et al.* 2007). DAT-1 is expressed in the eight dopaminergic neurons of *C. elegans*, where it has been shown to not only recycle extrasynaptic dopamine, but also to directly modulate neural function through a channel

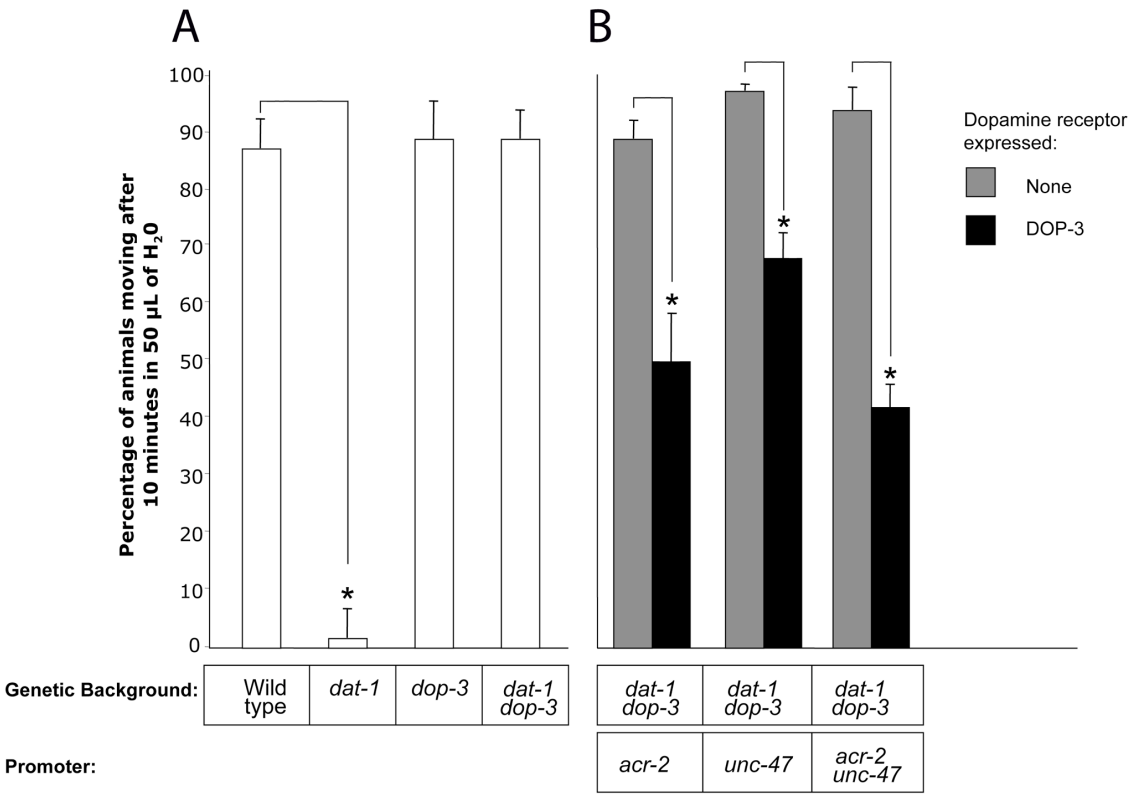


Figure 3.5. SWIP is a dopamine-dependent behavior regulated by DOP-3 in the cholinergic motor neurons. A.) The SWIP phenotype of *dat-1* mutants is rescued in the *dat-1; dop-3* double mutant, demonstrating that the SWIP response is mediated by excess signaling through the DOP-3 receptor. B.) Rescue of DOP-3 in either the cholinergic or GABAergic motor neurons with cell-specific promoters rescues the SWIP phenotype in a *dat-1; dop-3* double mutant background, demonstrating that DOP-3 is

mediating the SWIP response in the ventral cord motor neurons. *acr-2* is a cholinergic motor neuron specific promoter, and *unc-47* is a GABAergic motor neuron specific promoter. Bars represent the average of five trials of 10 L4 animals each for a total of 50 animals per strain. Error bars represent the 95% confidence interval. Asterisks represent statistical difference between two bars by Student t test ($p<0.005$).

mechanism (Jayanthi *et al.* 1998; Carvelli *et al.* 2004). In addition, it has also been shown that SWIP is mediated through the DOP-3 receptor, as the *dop-3(vs106)* mutation rescues the SWIP phenotype of *dat-1* animals (McDonald *et al.* 2007). These observations together suggest a model where in a *dat-1* mutant, extrasynaptic dopamine accumulates in the worm and over-stimulates DOP-3 receptors on the cholinergic motor neurons, leading to an inhibition of acetylcholine release and flaccid paralysis. We set out to test this model by performing tissue-specific rescue of DOP-3 in both the cholinergic and GABAergic motor neurons of *dat-1;dop-3* mutants, and our results confirm the model that DOP-3 is functioning in the cholinergic motor neurons to mediate SWIP (Fig. 3.5). These results establish the cholinergic motor neuron of *C. elegans* as a model system for the direct investigation of DOP-3 signaling and its role in acetylcholine release.

SWIP analysis of GPCR mutants and screen isolates support the model that dopamine is controlling acetylcholine release by modulating the excitability of the cholinergic motor neurons

Once we established that SWIP was a behavior mediated by DOP-3 signaling in the cholinergic motor neurons, we decided to investigate the role of various other components shown previously to be required for inhibition of acetylcholine release in the SWIP response (Fig. 3.6). The phenotype of the *cat-2::CAT-2* mutant provides further

evidence that SWIP is a dopamine-dependent behavior, as these animals over-express tyrosine hydroxylase (the rate limiting enzyme of dopamine biosynthesis) in the dopaminergic neurons and presumably have elevated levels of endogenous dopamine (Fig. 3.6*A*). Also, the *glr-1* and *ace-1* mutations partially rescue the SWIP phenotype of *dat-1* mutants, providing further evidence that DOP-3 receptor signaling is attenuating acetylcholine release, a conclusion supported further by the diminished SWIP response of *ace-2; ace-1; dat-1* triple mutants (Fig. 3.6*A*). Analysis of the effects of *goa-1, eat-16, dgk-1, and egl-30(*gf) mutations on the SWIP phenotype of dat-1 mutants demonstrates that these molecular components are inhibiting acetylcholine release in conjunction with or in parallel to DOP-3 in the cholinergic motor neurons (Fig. 3.6*B*). Interestingly, we also observed a partial SWIP phenotype in *dop-2(vs105)* mutants (Fig. 3.6*C*). We have established that SWIP is a phenotype caused by a DOP-3–mediated reduction in acetylcholine release, and this observation coupled with the expression of DOP-2 in the dopaminergic neurons (Suo *et al.* 2003) suggests that DOP-2 may function as an auto-receptor that negatively regulates the release of dopamine in response to high endogenous levels of the neurotransmitter. Finally, we also observed that the *dop-1(vs100)* mutation not only failed to rescue the SWIP phenotype of *dat-1* animals, but it also antagonized the effects of the *dop-3 (vs106)* mutation in the *dop-1 dop-3; dat-1* triple mutant (Fig. 3.6*C*). These results suggest that DOP-1 may be antagonizing DOP-3 receptor signaling in the cholinergic motor neurons; however, as DOP-1 is expressed in many neurons in *C. elegans*, additional experiments are necessary to demonstrate that this antagonism is in fact occurring in the same cell.

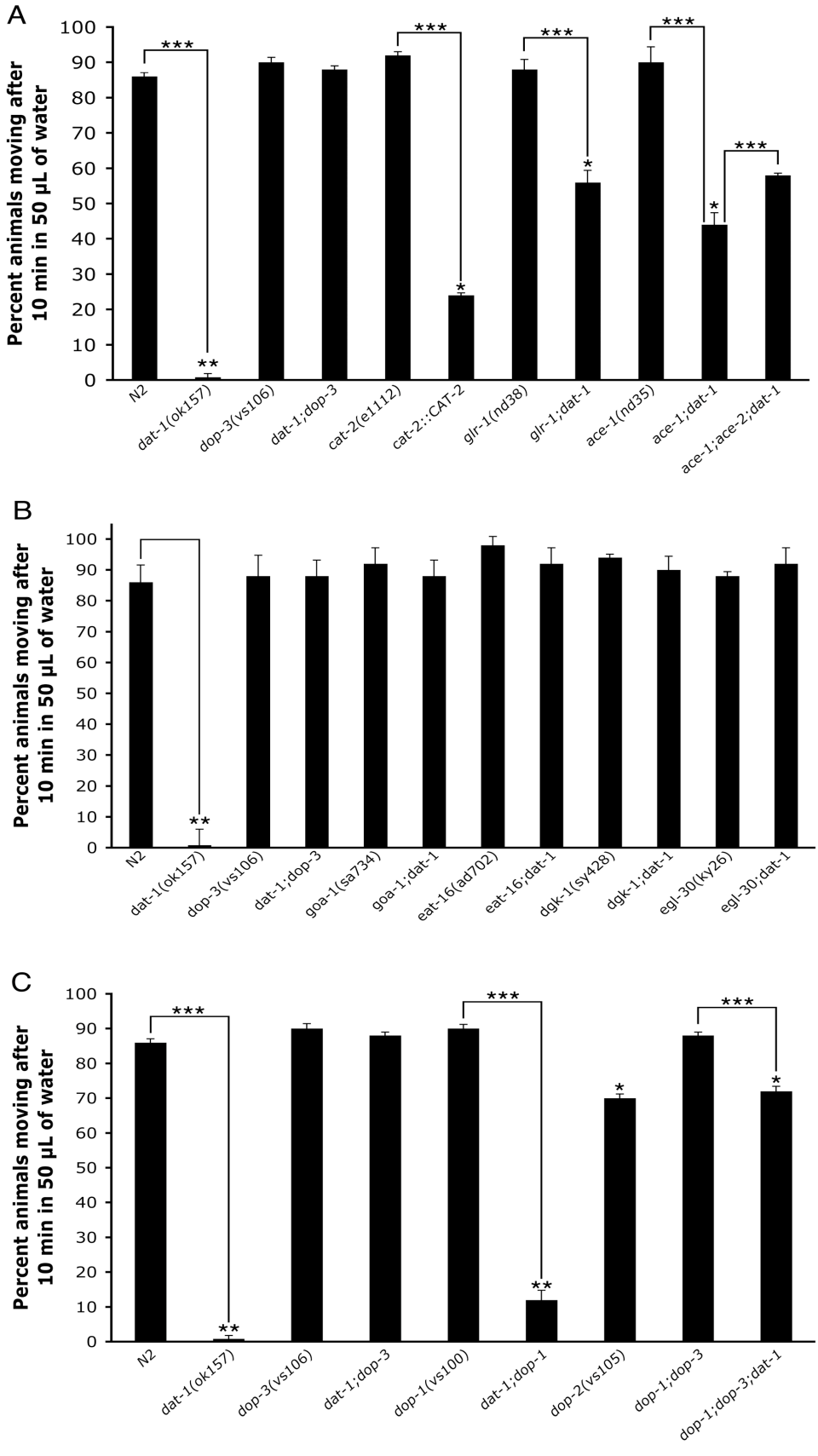

A
Percent animals moving after 10 min in 50 µL of water
100
90
80
70
60
50
40
30
20
10
0
***
**
*
N2
dat-1(ok157)
dop-3(vs106)
dat-1;dop-3
cat-2(e1112)
cat-2::CAT-2
glr-1(nd38)
glr-1;dat-1
ace-1(nd35)
ace-1;dat-1
ace-1;ace-2;dat-1
B
Percent animals moving after 10 min in 50 µL of water
N2
dat-1(ok157)
dop-3(vs106)
dat-1;dop-3
goa-1(sa734)
goa-1;dat-1
eat-16(ad702)
eat-16;dat-1
dgk-1(sy428)
dgk-1;dat-1
egl-30(ky26)
egl-30;dat-1
C
Percent animals moving after 10 min in 50 µL of water
N2
dat-1(ok157)
dop-3(vs106)
dat-1;dop-3
dop-1(vs100)
dat-1;dop-1
dop-2(vs105)
dop-1;dop-3
dop-1;dop-3;dat-1

Figure 3.6. The SWIP phenotype is the result of excess acetylcholine release from the cholinergic motor neurons. A.) Animals over-expressing the tyrosine hydroxylase CAT-2 (the rate-limiting enzyme in dopamine biosynthesis) exhibit a strong SWIP phenotype, demonstrating that SWIP is mediated by excess dopamine signaling. *glr-1(nd38)* and *ace-1(nd35)* mutants both partially fail to rescue the SWIP response in a *dat-1* mutant background, demonstrating that SWIP depends on decreased acetylcholine release from the cholinergic motor neurons. This observation is further supported by the less severe phenotype of the *ace-1; ace-2; dat-1* triple. B.) Mutants in inhibitory components of dopamine signaling rescue the SWIP response in a *dat-1* mutant background, demonstrating that these components are influencing dopaminergic control of acetylcholine release in the cholinergic motor neurons. C.) The *dop-1(vs100)* mutation fails to rescue the SWIP phenotype of *dat-1* mutants, but antagonizes the wild-type phenotype of *dat-1;dop-3* double mutants, suggesting that DOP-1 may antagonize DOP-3 receptor signaling in the cholinergic motor neurons. Also, *dop-2* mutants exhibit partial SWIP, suggesting that DOP-2 may function as an auto-receptor that down-regulates dopamine release. Bars represent the average of five trials of 10 L4 animals each for a total of 50 animals per strain. Error bars represent the 95% confidence interval. Asterisks represent statistical difference from N2 by Student t test (* = $p<0.005$, **=$p<0.001$) and the statistical difference between two bracketed bars (*** = $p<0.005$).

DOP-1 and DOP-3 act antagonistically in the cholinergic motor neurons to modulate the release of acetylcholine

As a final confirmation of the role of DOP-3 in controlling acetylcholine release from the cholinergic motor neurons, we conducted a genetic analysis of acetylcholine release using acute exposure to the acetylcholinesterase aldicarb. Aldicarb is a well-established paradigm for the analysis of neuromuscular junction activity in *C. elegans*, as wild type animals become paralyzed in response to aldicarb in a dose-dependent manner (Nguyen *et al.* 1995). Mutants that exhibit increased acetylcholine release are sensitive to the paralytic effects of aldicarb, whereas mutants that release diminished levels of acetylcholine exhibit aldicarb resistance (Mahoney *et al.* 2006). Therefore, conclusions can be drawn about a mutation's effect on acetylcholine release based on its response to aldicarb, establishing aldicarb response as a powerful genetic tool to identify and characterize components involved in neurotransmitter release.

If dopamine signaling is controlling acetylcholine release, *dop*-3, *ace-1*, and *glr-1* mutants are all expected to exhibit sensitivity to the paralytic effects of aldicarb. In addition, previous studies have used aldicarb sensitivity as a means to conduct tissue-specific rescue experiments (Lackner *et al.* 1999), an approach that we decided to implement to directly investigate the antagonism of DOP-1 and DOP-3 receptor signaling in the cholinergic motor neurons.

Surprisingly, we did not observe significant sensitivity of either *ace-1* or *dop-3* mutants to 1 mM aldicarb exposure, although sensitivity was observed for *glr-1* mutants (Fig. 3.7*A*). While these results are consistent with the model of dopamine signaling acting to exert subtle modulation of neurotransmission (Bam ford *et al.* 2004), we decided to adopt a more sensitive assay to investigate the cholinergic activity of both *ace-1* and *dop-3* mutants, and in fact both mutants were observed to exhibit aldicarb sensitivity in this assay (see Appendix A). In addition, the *ace-1 dop-3* double mutant is sensitive to acute aldicarb exposure, demonstrating an additive effect of these two mutations that increases acetylcholine release (Fig. 3.7*A*). Furthermore, *ace-1; ace-2* mutants show enhanced sensitivity to aldicarb as compared to either single mutant (Fig. 3.7*A*), supporting the conclusion that *ace-1* and *dop-3* mutants do in fact exhibit increased cholinergic activity.

The sensitivity of the *glr-1* mutation to aldicarb is most likely caused by an over-stimulation of the cholinergic motor neurons upstream of the DOP-3 receptors. In the *glr-1* mutant, DOP-3 is still presumably functioning to inhibit acetylcholine release despite the over-stimulation the neuron is receiving from the constitutively-active command interneurons in these mutants. Thus, we reasoned that the *dop-3(vs106)*

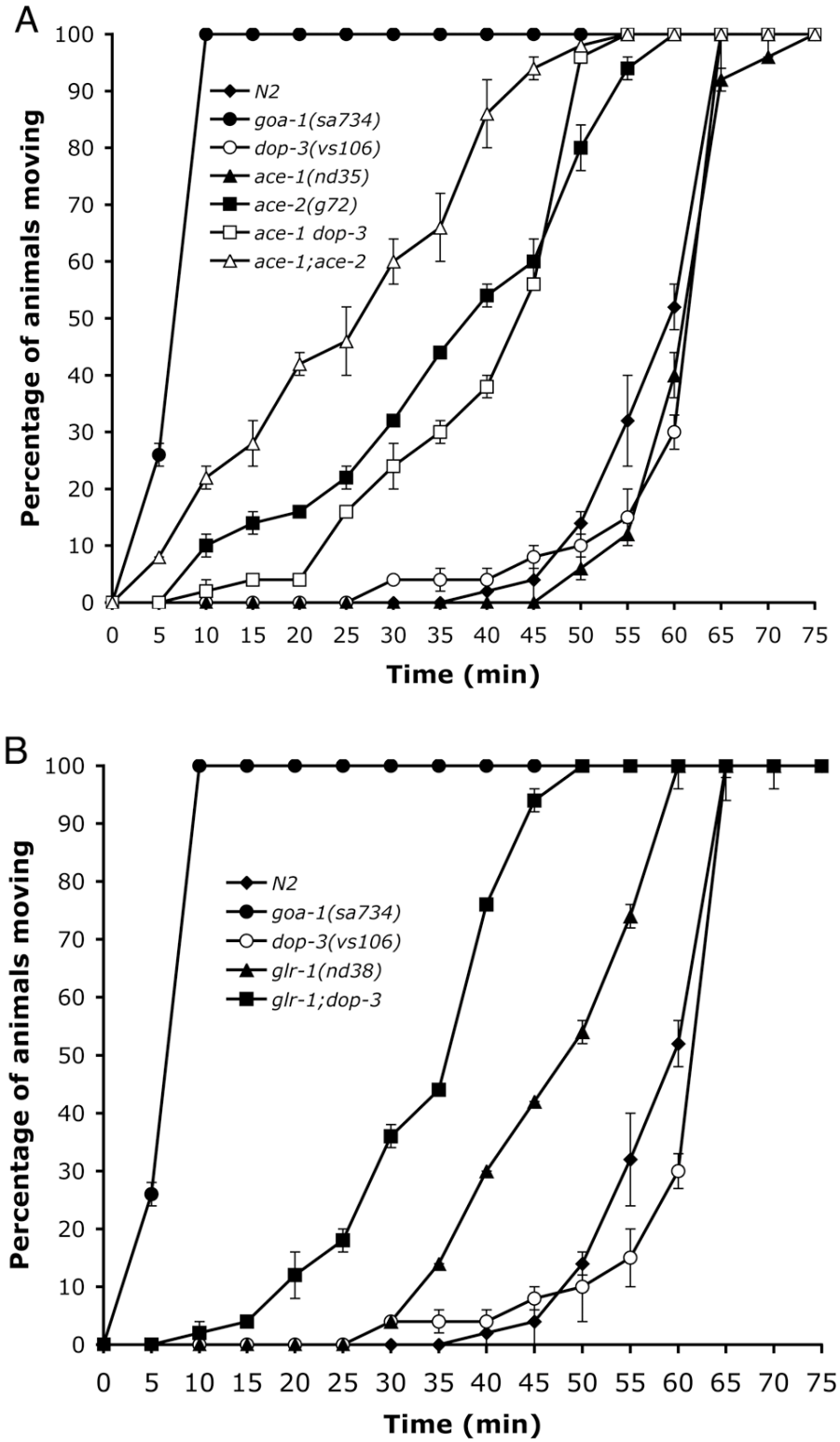
mutation should further increase the aldicarb sensitivity of *glr-1* mutants, and when tested, the *glr-1; dop-3* double mutant is more sensitive to aldicarb than *glr-1* mutants alone, demonstrating that DOP-3 is in fact acting to inhibit acetylcholine release from the cholinergic motor neurons (Fig. 3.7*B*).

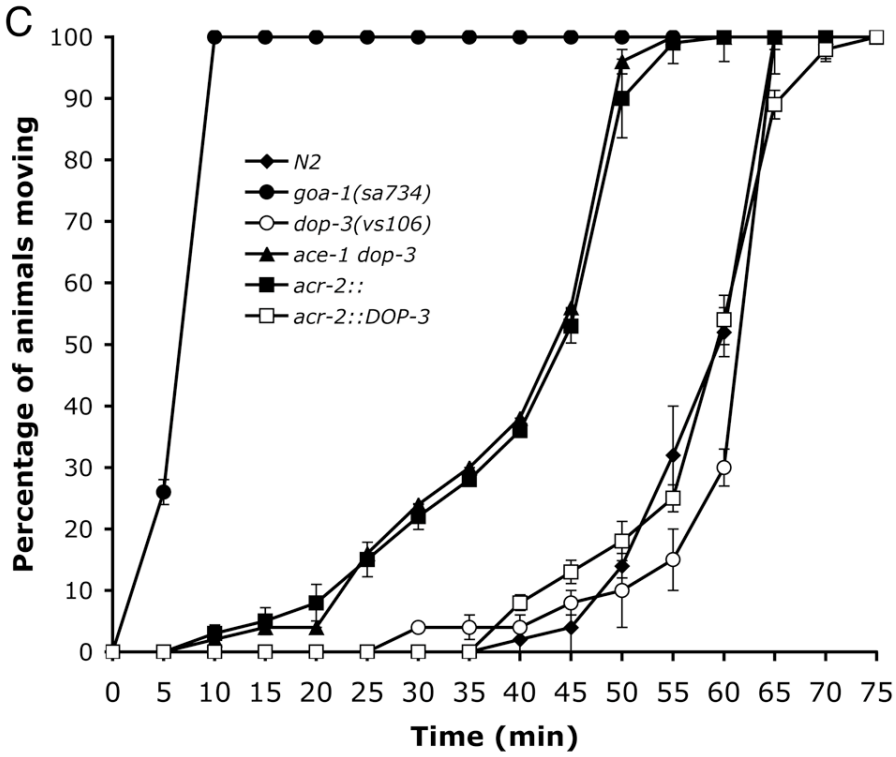
To demonstrate that DOP-3 is inhibiting acetylcholine release in the cholinergic motor neurons, we rescued DOP-3 in *ace-1 dop-3* double mutants using the cholinergic motor neuron-specific promoter *acr-2* (Hallam *et al.* 2000) and observed their response to aldicarb (Fig. 3.7*C*). These transgenic animals exhibit the wild-type level of aldicarb sensitivity seen in *ace-1* single mutants, demonstrating that DOP-3 is inhibiting acetylcholine release specifically in the cholinergic motor neurons.

Once it was established that DOP-3 was acting specifically in the cholinergic motor neurons to inhibit acetylcholine release, we decided to address the role of DOP-1 receptor signaling in acetylcholine release using aldicarb sensitivity. To investigate the issue of DOP-1 and DOP-3 antagonism in the cholinergic motor neurons, we first chose to observe the aldicarb sensitivity of *ace-1 dop-1 dop-3* triple mutants (Fig. 3.7*C*). If DOP-1 antagonizes DOP-3 receptor signaling, then *ace-1 dop-1 dop-3* mutants should exhibit wild-type resistance to aldicarb, as the effects of the *dop-1* mutation should counteract the additive effects of the *dop-3* and *ace-1* mutations on acetylcholine release. Analysis of these mutants supports this model, demonstrating that DOP-1 does in fact antagonize DOP-3 receptor signaling and its effects on acetylcholine release.

Next, we sought to demonstrate that DOP-1 antagonizes DOP-3-mediated inhibition of acetylcholine release in the cholinergic motor neurons, a hypothesis supported by the co-localization of DOP-1 and DOP-3 to these cells and cell-specific

rescue of exogenous dopamine resistance (Chase *et al.* 2004). To answer this question, we created transgenic *ace-1 dop-1 dop-3* animals expressing DOP-1 under the expression of the cholinergic motor neuron-specific promoter *acr-2* and observed their sensitivity to aldicarb (Fig. 3.7*C*). If DOP-1 is antagonizing DOP-3 signaling in the cholinergic motor neurons, then rescue of DOP-1 in these neurons of *ace-1 dop-1 dop-3* mutants should increase the aldicarb sensitivity of these animals by relieving the antagonistic effect of the *dop-1* mutation on the additive effects of the *ace-1* and *dop-3* mutations on acetylcholine release. Analysis of these mutants supports this model, demonstrating that DOP-1 and DOP-3 have antagonistic effects on acetylcholine release in a single cell: the cholinergic motor neuron.


A
Percentage of animals moving
Time (min)
N2
goa-1(sa734)
dop-3(vs106)
ace-1(nd35)
ace-2(g72)
ace-1 dop-3
ace-1;ace-2
B
Percentage of animals moving
Time (min)
N2
goa-1(sa734)
dop-3(vs106)
glr-1(nd38)
glr-1;dop-3


C
Percentage of animals moving
Time (min)
N2
goa-1(sa734)
dop-3(vs106)
ace-1 dop-3
acr-2::
acr-2::DOP-3

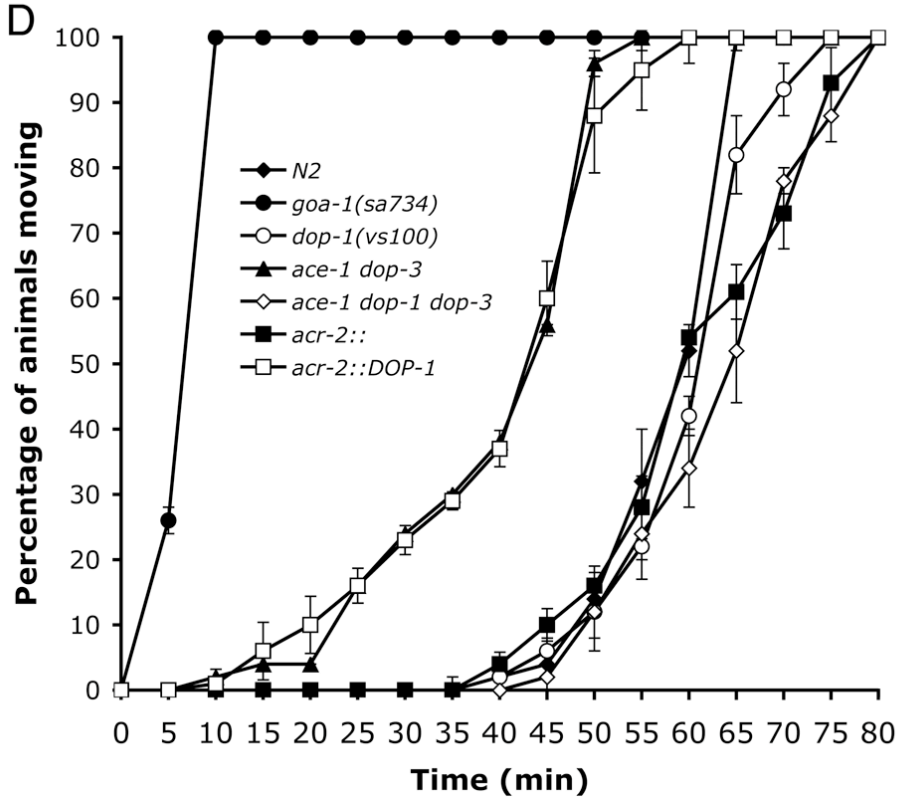

D
Percentage of animals moving
Time (min)
N2
goa-1(sa734)
dop-1(vs100)
ace-1 dop-3
ace-1 dop-1 dop-3
acr-2::
acr-2::DOP-1

Figure 3.7. DOP-1 and DOP-3 act antagonistically in the cholinergic motor neurons to modulate the release of acetylcholine. A.) Mutants with increased levels of acetylcholine release are sensitive to the paralytic effects of the acetylcholinesterase inhibitor aldicarb. Surprisingly, *dop-3* and *ace-1* mutants that have been shown to exhibit increased acetylcholine release do not show sensitivity to aldicarb. However, *dop-3 ace-1* double mutants exhibits aldicarb sensitivity, demonstrating an additive effect of these mutations on acetylcholine release. Also, a similar additive effect can be seen in the *ace2; ace-1* double mutant. B.) The sensitivity of *glr-1(nd35)* mutants (which exhibit constitutive activation of the cholinergic motor neurons) is exacerbated in the *glr-1; dop-3* double mutant, demonstrating that DOP-3 functions to inhibit the release of acetylcholine from the cholinergic motor neurons. C.) Expression of DOP-3 in the cholinergic motor neurons of *dop-3 ace-1* animals rescues the aldicarb resistance of these animals to wild-type levels, demonstrating that DOP-3 is regulating acetylcholine release in the cholinergic motor neurons. D.) Expression of DOP-1 in the cholinergic motor neurons of *dop-3 dop-1 ace-1* animals rescues the aldicarb sensitivity of these animals to that of the *dop-3 ace-1* double mutant, demonstrating that signaling through the DOP-1 receptor is antagonizing DOP-3 signaling in the cholinergic motor neurons to modulate acetylcholine release. n=25 young adult animals per strain. Assays were done in duplicate for a total of 50 animals per strain. Error bars represent standard error of the mean.

tbc-4 mutants are defective in the exogenous dopamine response

During the process of mapping the screen isolate later identified as *ace-1*, we identified another gene involved in dopamine response. Using intra-chromosomal SNP mapping, we mapped the position of the mutation to less than 2 cam on the far right end of the X chromosome. However, mapping genes located at the ends of chromosomes is complicated by the paucity of visible genetic markers available for use in three-factor genetic mapping. As a means to circumvent this problem, we adopted a candidate approach to map the mutation: we identified potential candidate genes from the *C. elegans* genome based on both their location near the end of chromosome X and their predicted biological function. We then obtained deletion mutants of these genes and examined their sensitivity to exogenous dopamine. A mutation in one of these genes, the putative Rab GTPase activating protein *tbc-4*, caused resistance to exogenous dopamine

(Fig. 3.8*A*), suggesting that it may be allelic to the mutation isolated in the screen. However, the results of genetic complementation between the two mutations demonstrated that they were non-allelic and thus mutations in two separate genes. Although *ace-1* was later mapped and cloned using two-factor genetic mapping, the candidate approach was successful in identifying *tbc*-4 as a novel component in dopamine response.

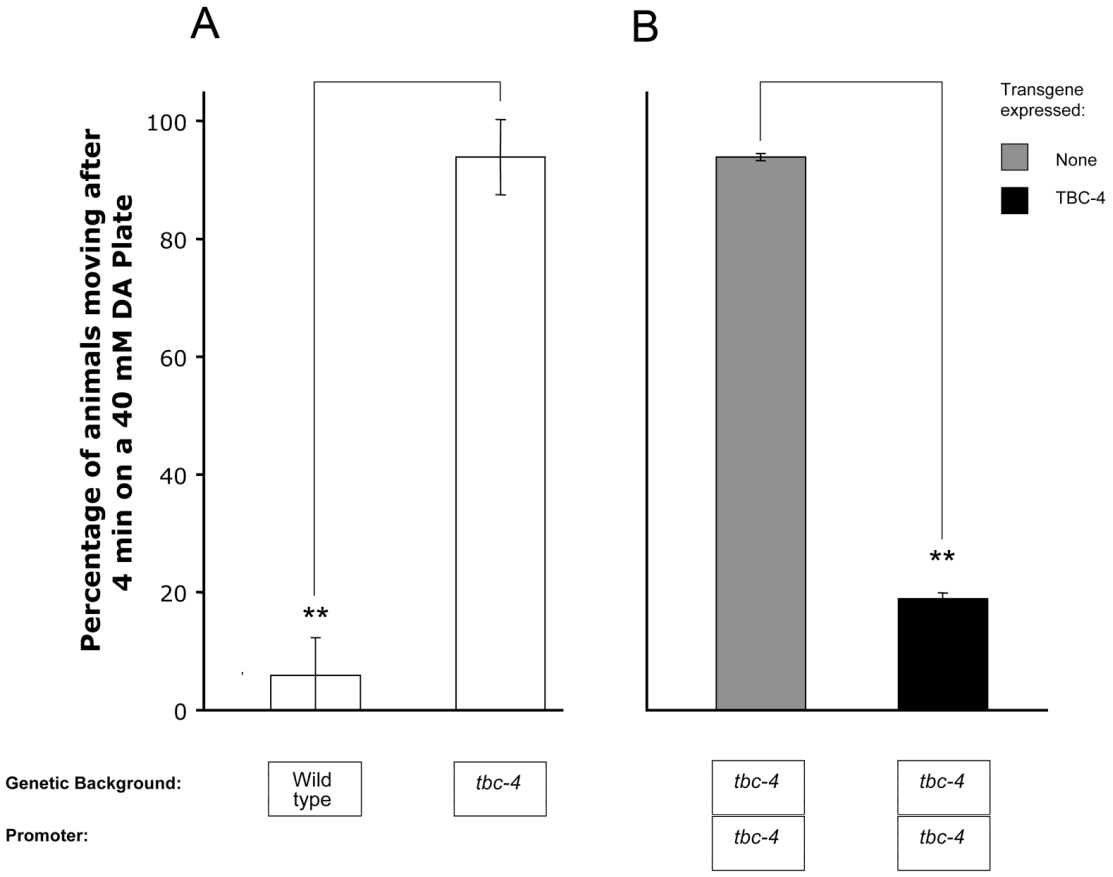


Figure 3.8. The dopamine resistance of *tbc-4* mutants can be rescued by TBC-4 under the expression of its own promoter. A.) *tbc-4(ok3041)* mutants are resistant to exogenous dopamine. B.) Transgenic *tbc-4* mutants expressing TBC-4 under its own promoter are no longer resistant to exogenous dopamine, demonstrating that the dopamine resistance seen in *tbc-4(ok3041)* mutants is in fact a result of the loss of TBC-4 function. White bars represent the average of five trials of 10 young adult animals for a total of 50 animals per strain. Colored bars represent the average of five trials of 10 young adults for each of five transgenic lines for a total of 250 animals per bar. Asterisks represent the difference between two bars by Student t test ($p<0.005$).

To further demonstrate that TBC-4 was in fact required for exogenous dopamine response, we generated transgenic *tbc-4* animals expressing TBC-4 under its own promoter and assayed these animals for dopamine sensitivity (Fig. 3.8*B*). The rescue of dopamine sensitivity in these transgenic animals illustrates that TBC-4 is in fact required for sensitivity to exogenous dopamine and suggests a role for Rab-mediated vesicular trafficking in facilitating dopaminergic control of locomotion.

TBC-4 closely resembles the mammalian Rab GAP EPI64

Once we had established that TBC-4 is likely playing a role in dopamine signaling, we decided to investigate the likely targets and action of TBC-4 in *C. elegans*. Preliminary sequence analysis verified the presence of a TBC domain in the TBC-4 amino acid sequence (Fig. 3.9), a domain conserved in all previously characterized Rab GAPs from yeast to man (Bernard's 2003) with one notable exception (Fukui *et al.* 1997). This sequence analysis also revealed the absence of any additional conserved domains, which presumably play a role in defining the specificity and function of Rab GAPs to one or a subset of Rabs. However, these observations allowed a comparison of TBC-4 to Rab GAPs in other species (Fig. 3.9), which revealed that TBC-4 closely resembles the mammalian Rab GAP EPI64, which has been shown to exhibit GAP activity specifically towards the AEX-6 homolog Rab27A in a melanocytes (Itoh and Fukuda 2007).

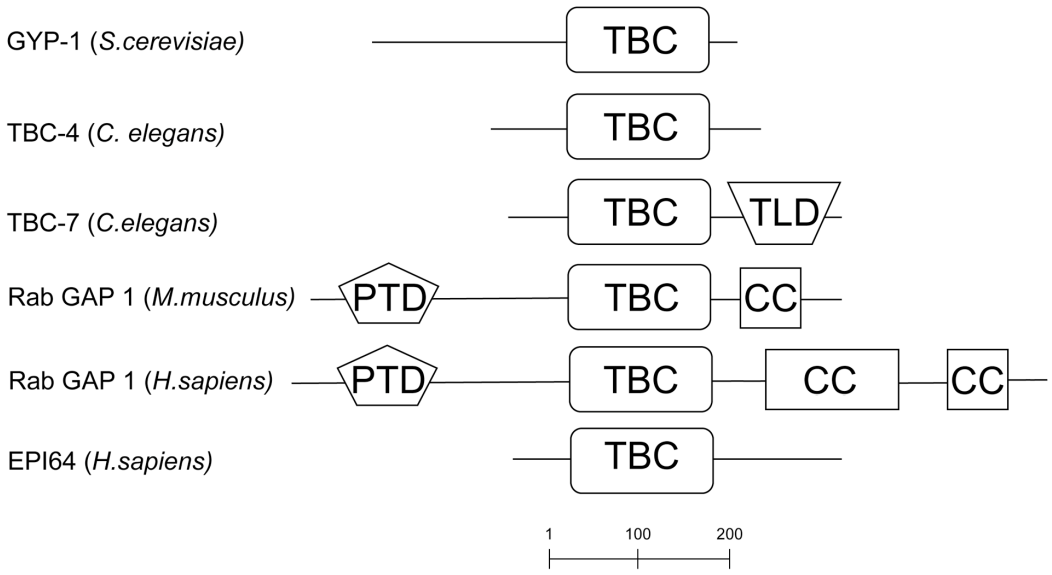


Figure 3.9. Schematic comparison of the domain compositions of several Rab GAPs suggest that TBC-4 most closely resembles the human Rab GAP EPI64. The amino acid sequences of several Rab GAPs were selected and their domain structure was determined using the Protein SMART online server. All sequences selected contained the TBC domain stereotypical of Rab GAP proteins and were diagramed to illustrate the variety of Rab GAP domain structures both within and across species. TBC: Tre-2/Bub2/Cdc16 domain. PTD: Phosphotyrosine-binding domain. CC: Coiled-coil domain. TLD: Domain in TBC and LysM-containing proteins. Scale represents an approximate number of amino acid residues.

A subsequent alignment of the TBC-4 amino acid sequence with several other closely related Rab GAPs further suggests that TBC-4 is most similar to EPI64 and may function in the same capacity as a Rab GAP for AEX-6 (Fig. 3.10). However, these observations do not exclude the possibility that TBC-4 exhibits GAP activity towards several Rabs, as several regulators of Rab activity have been shown to modify the activity of more than one Rab (Mahoney *et al.* 2006, Ishibashi *et al.* 2008).

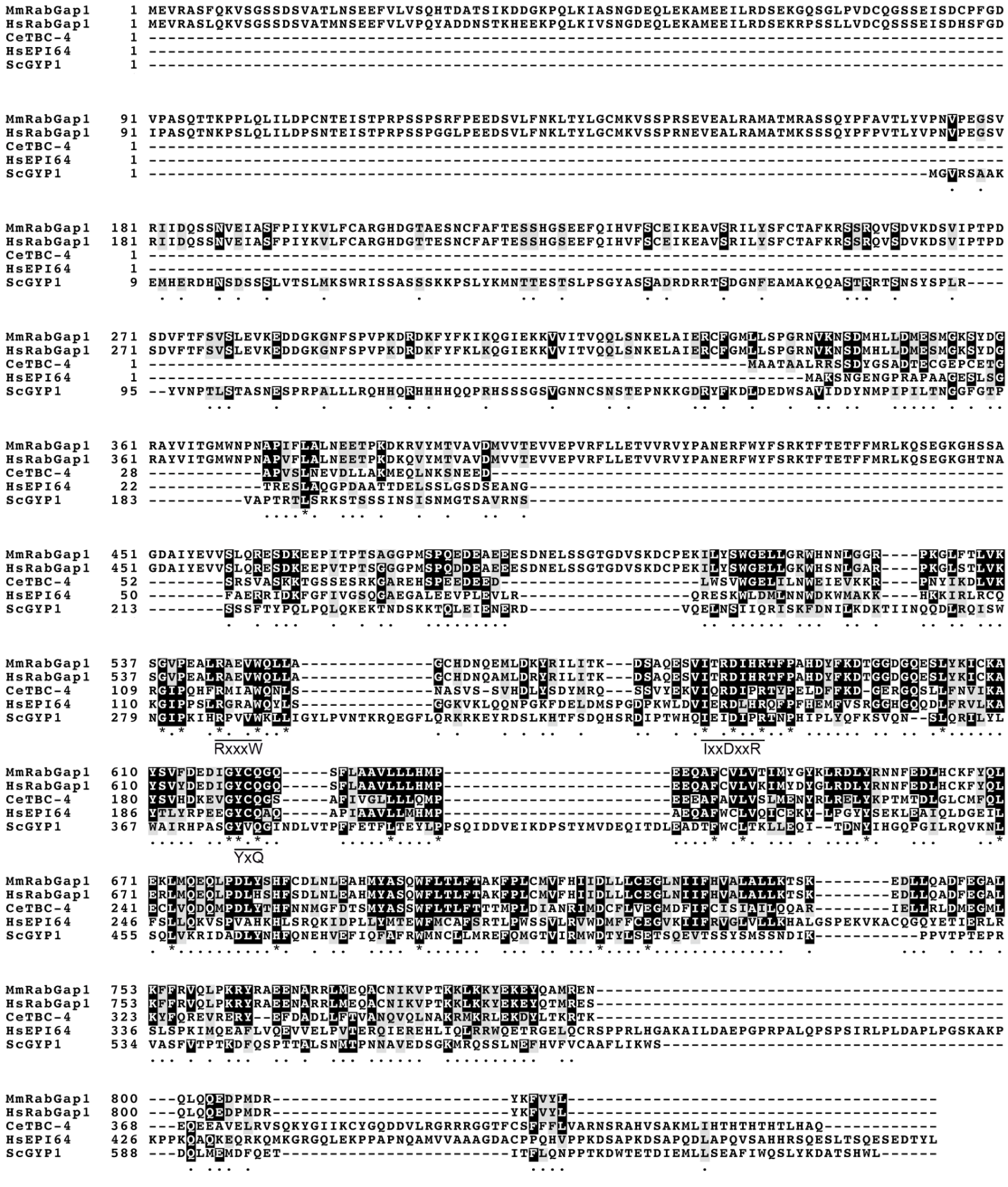


Figure 3.10. Sequence alignment of selected Rab GAPs demonstrates that TBC-4 is most similar to EPI64. Despite the conservation across all selected Rab GAPs of the TBC domain critical for GAP activity, TBC-4 shares a higher degree of similarity with EPI64. ClustalX was used to align the amino acid sequence of several Rab GAPs. Shading was done using the online BoxShade server. Black shading indicates identical residues. Grey shading indicates similar residues. The three conserved motifs essential for GAP function (RxxxW, IxxDxxR, and YxQ) (Pan *et al.* 2006) are indicated below the alignment with a solid line.

TBC-4 is expressed in the nervous system of *C. elegans* and co-localizes with RAB-3 in the cholinergic motor neurons

To further characterize the role of TBC-4 in neurotransmission, we determined the expression pattern of TBC-4 by observing the florescence of transgenic animals carrying a *tbc-4::*GFP reporter (Fig. 3.11). The expression pattern of TBC-4 has been previously reported, but the promoter used in that study included only 2.2 kb of the first intron of the *tbc-4* ORF (Hunt-Newbury *et al.* 2007). In our study, we used the first 9 kb of 5' upstream regulatory sequence in addition to the first 3.2 kb of the *tbc-4* ORF (including the first exon and the entire first intron) as the promoter to drive GFP expression (Fig. 3.11*A*). By using this approach, we determined that TBC-4 is expressed in the pharyngeal muscle, ventral cord motor neurons, and select neurons of the head and tail (Fig. 3.11*B*), an expression pattern consistent with those of both RAB-3 and AEX-6 (Nonet *et al.* 1997; Mahoney *et al.* 2006). Subsequent analysis revealed that TBC-4 was likely expressed in the neurons of the tail that regulate defecation (Fig. 3.11*C*), a behavior previously established to be mediated by AEX-6 (Thomas 1990). In addition, TBC-4 expression was also observed in the vulval muscle (Fig. 3.11*D*) post-synaptic to the hermaphrodite specific neurons where RAB-3 has been previously shown to localize (Patel *et al.* 2006). This data demonstrates that TBC-4 is expressed throughout the nervous system of *C. elegans* in a pattern consistent with that of RAB-3 and AEX-6 and therefore suggests the possibility of TBC-4 acting in the same cells to regulate the activity of these Rabs. To further investigate the possibility of TBC-4 regulating the activity of presynaptic Rabs, we analyzed the co-expression of *tbc-4::*GFP with

florescent reporters for both DOP-1, a marker for the cholinergic motor neurons, and RAB-3 (Fig. 3.12).

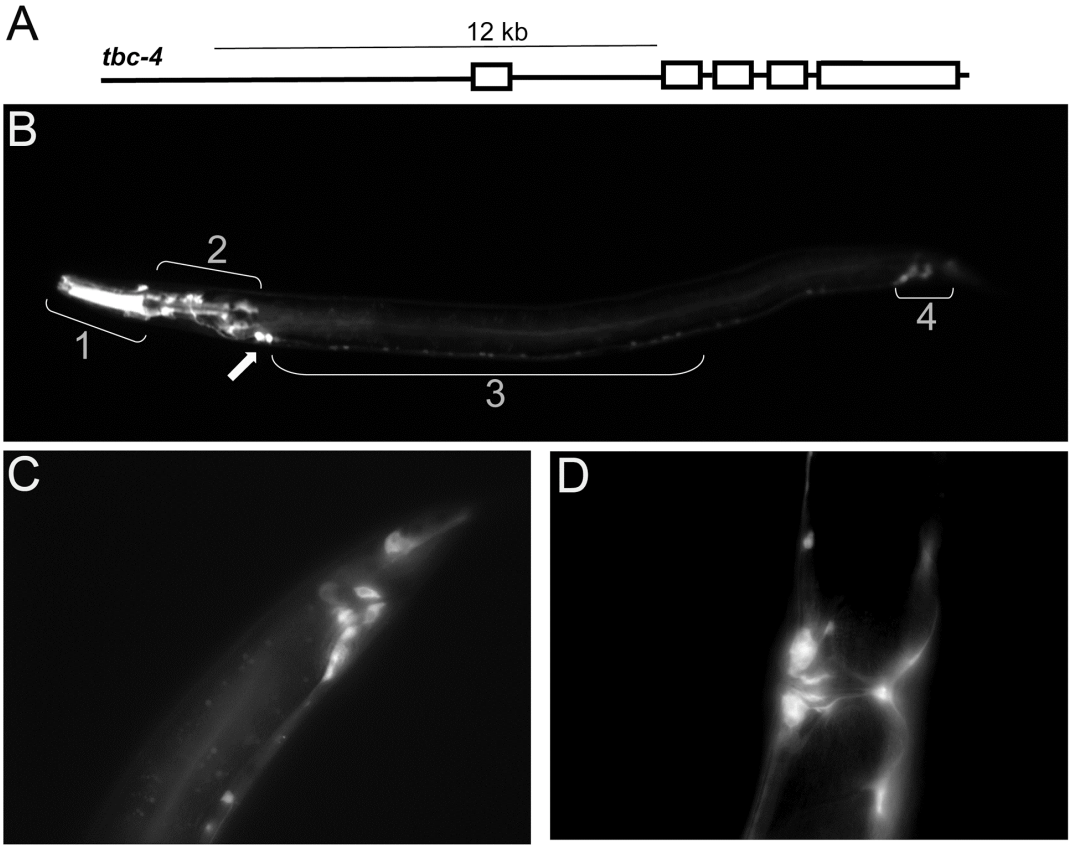


Figure 3.11. TBC-4 is expressed in the nervous system of C. elegans. A.) A GFP reporter construct was generated using a 12 kb promoter (including the first intron) amplified from genomic DNA and fused to a fragment amplified from pPD49.77 containing the GFP coding sequence and the unc-54 3' UTR using PCR fusion. 50 ng/μL of the PCR product was co-injected with 50 ng/μL of the L15EK rescue construct into *lin-15(n765ts)* animals and transgenic lines were isolated by the absence of the MuV phenotype at 20º. B.) TBC-4 is expressed in the pharyngeal muscle (1), the nerve ring (2), the ventral nerve cord (3), and neurons of the tail (4). Arrow indicates two unidentified interneurons that may be command interneurons. C.) TBC-4 expression is seen in several neurons of the tail which may regulate defecation in C. elegans. D.) TBC-4 is expressed in vulval muscle. All images taken with a Cool Snap EZ camera on a Nikon Eclipse FN1 equipped with epiflouresence using either a 10x (B) or 60x oil immersion objective. All imaged animals were immobilized with 2mM levamisole and mounted on microscope slides containing 2% agarose.

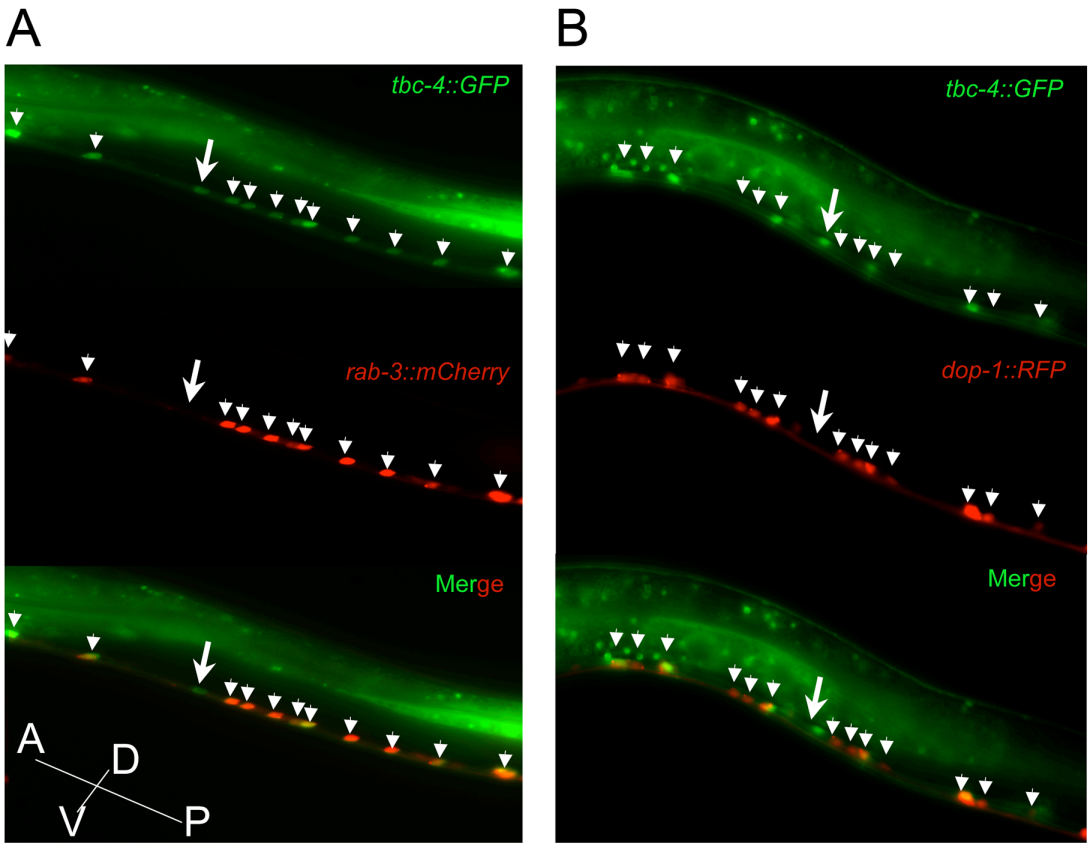


Figure 3.12. TBC-4 is co-expressed with RAB-3 in the cholinergic motor neurons. A.) *tbc-4::*GFP expression largely overlaps with *rab-3::*mCherry expression in animals expressing *tbc-4::gfp* and *rab-3::mcherry*, suggesting the possibility of a direct interaction between TBC-4 and RAB-3. B.) *tbc-4::*GFP expression largely overlaps with *dop-1::*RFP expression in the cholinergic motor neurons of transgenic animals expressing both *tbc-4::gfp* and *dop-1::rfp*, which demonstrates that TBC-4 is expressed in the cholinergic motor neurons. In both (A) and (B), *tbc-4::*GFP is expressed in cells other than those expressing either *dop-1::*RFP or *rab-3::*mCherry, suggesting that TBC-4 may be expressed in cells other than the cholinergic motor neurons. All images taken with a Cool Snap EZ camera on a Nikon Eclipse FN1 microscope equipped with epiflouresence using a 60x oil immersion objective. All imaged animals were immobilized with 2mM levamisole and mounted on microscope slides containing 2% agarose. Merged images were created using Nikon Elements imaging software.

As a result of this analysis, we have determined that TBC-4 co-localizes with both DOP-1 and RAB-3 in the cholinergic motor neurons of the ventral nerve cord (Fig. 3.12), and as RAB-3 and RAB-27 have been shown to be co-localized in these cells, this data

suggests that TBC-4 may act in these neurons to regulate locomotion by regulating the activity of a presynaptic Rab. In addition, we also observed neurons expressing *tbc-4*::GFP in the ventral nerve cord that did not overlap with *dop-1*::RFP expression. As the *dop-1::*RFP transgene used in this study was integrated using UV/TMP-associated integration (), all cells in these transgenic animals that express DOP-1 will also express RFP, and as DOP-1 is expressed in all of the cholinergic motor neurons of the ventral nerve cord, the presence of *tbc-4::*GFP-expressing cells in the ventral cord that do not co-localize with *dop-1::*RFP (Fig. 3.12*B*, large arrow) demonstrates that TBC-4 must be expressed in additional cells of the ventral cord. These cells are likely GABAergic motor neurons, a hypothesis supported by the localization of the GABAergic motor neurons in the ventral nerve cord (White *et al.* 1986, Schuske *et al.* 2004). This observation is intriguing, as DOP-3 expression has also been shown in both the cholinergic and GABAergic motor neurons of the ventral cord (Chase *et al.* 2004), further suggesting a possible role for TBC-4 in dopaminergic regulation of locomotion.

TBC-4 decreases acetylcholine release and antagonizes RAB-3

Sequence and expression analysis have identified TBC-4 as a putative regulator of Rab activity at the presynaptic terminal, and the resistance of *tbc-4* mutants to exogenous dopamine suggests that TBC-4 is playing a role in regulating dopaminergic neurotransmission. As we have established that dopamine signaling controls locomotion by regulating the release of acetylcholine from the presynaptic terminals of cholinergic motor neurons, we sought to observe the role of TBC-4 in acetylcholine release through a behavioral analysis of the *tbc-4(ok3041)* mutant.

Egg laying in *C. elegans* is a well-characterized behavior that is stimulated by serotonin release from the hermaphrodite-specific neurons (HSNs) and inhibited by acetylcholine release onto G-protein coupled acetylcholine receptors expressed on the HSNs, and mutants with increased acetylcholine release have been shown to hold more eggs *in utero* as compared to the wild-type (Bany *et al.* 2003, Fig. 3.13*A*) which establishes egg laying as a behavioral read-out of endogenous acetylcholine release. As a preliminary assessment of the role of TBC-4 in acetylcholine release, we sought to observe the effects of the *tbc-4(ok3041)* mutation on egg laying behavior (Fig. 3.13*B*).

Our results indicate that while *tbc-4* mutants exhibit wild-type egg laying behavior, *dop-3 tbc-4* mutants are defective in egg laying, which demonstrates an additive effect of these genes' negative regulation of acetylcholine release in the egg laying circuit. This observation is supported by previous evidence that dopamine inhibits egg laying through D2-like receptors (Weinshenker *et al.* 1995). In addition, the *tbc-4(ok3041)* mutation antagonizes the egg laying defect of *rab-3* mutants. RAB-3 has been shown to be expressed at the presynapse of the HSNs (Patel *et al.* 2006) and as we have shown that TBC-4 is expressed in the adjacent vulval muscle innervated by the HSNs, it is plausible that TBC-4 may negatively regulate RAB-3-mediated control of neurotransmitter release in the HSNs.

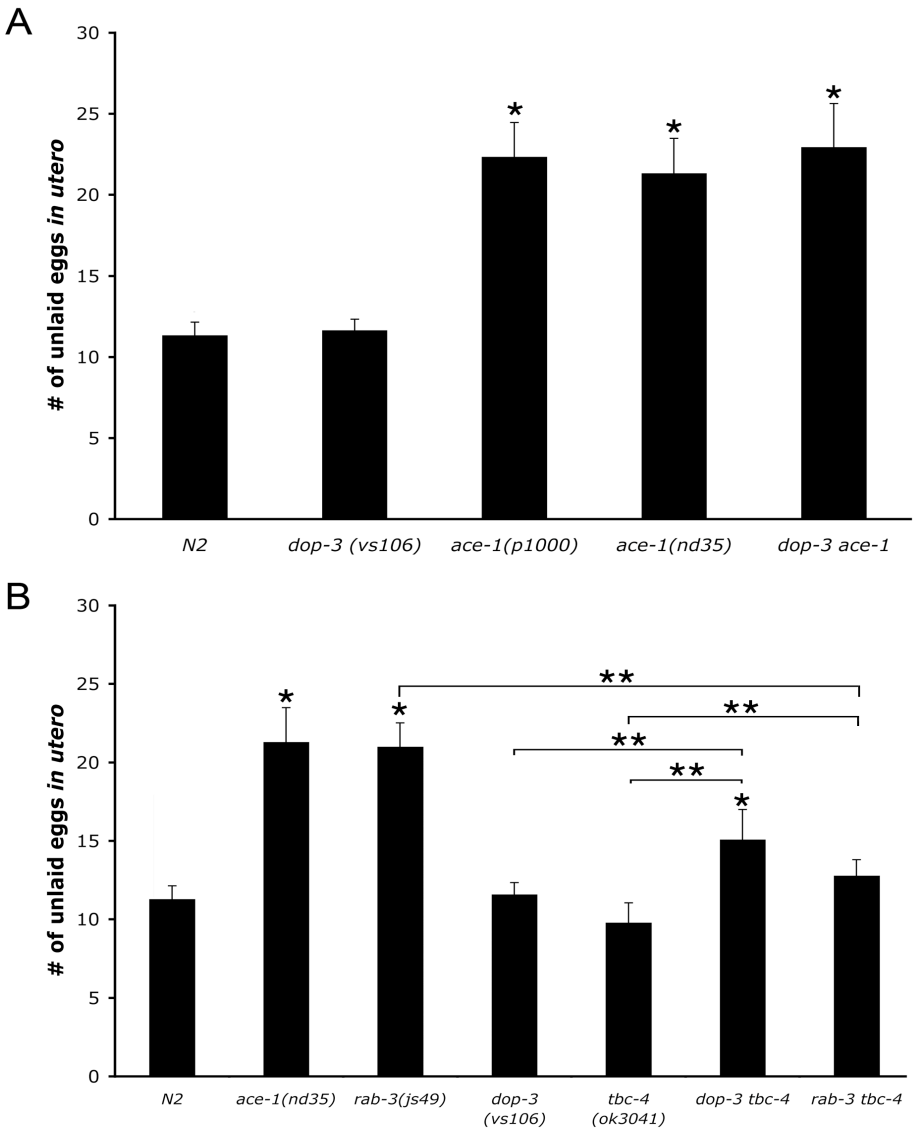


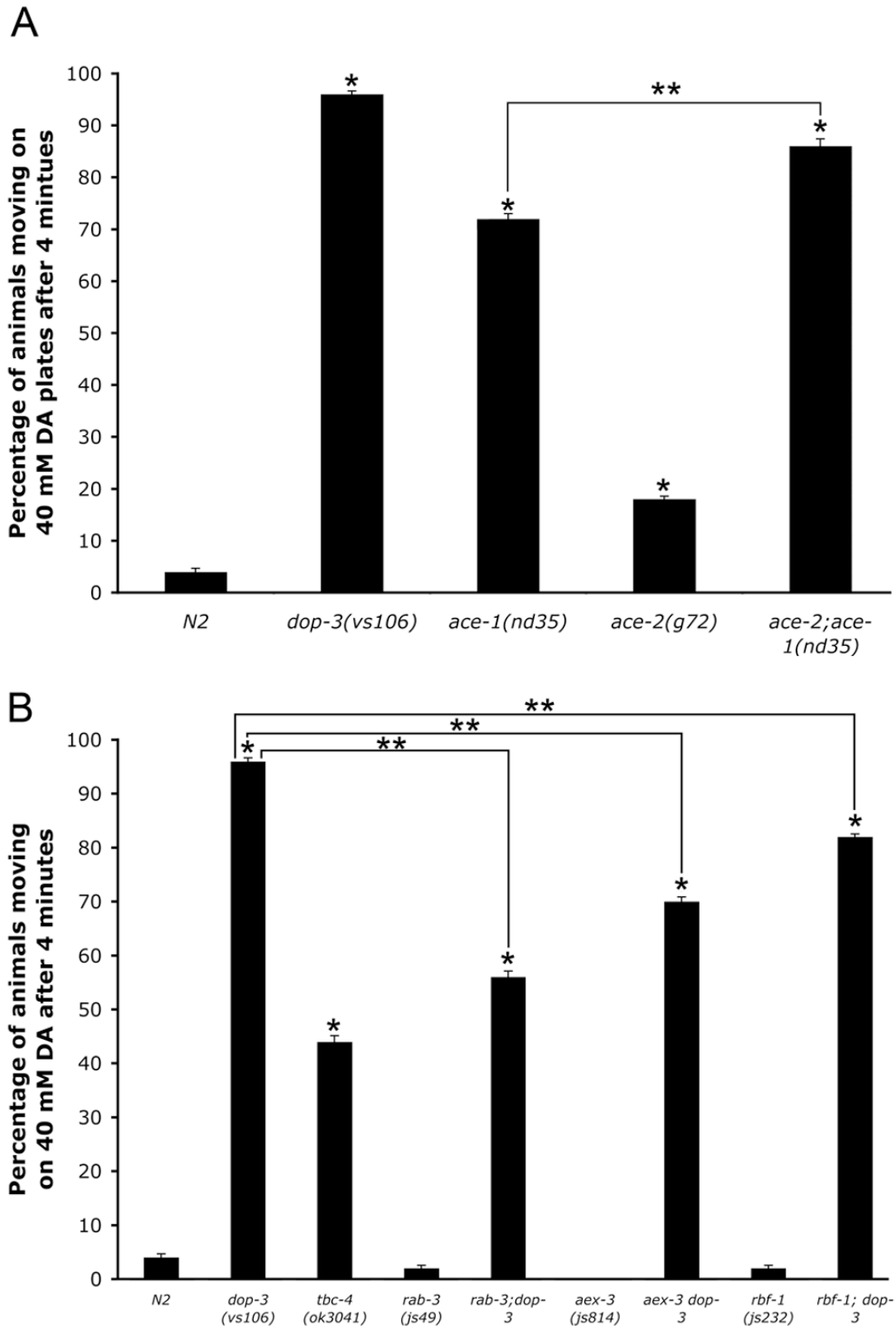
Figure 3.13. Mutations in dopamine signaling and vesicular trafficking components have additive or antagonistic effects on acetylcholine release. A.) While *ace-1* mutants exhibit moderate egg-laying defects, *dop-3* mutants show no phenotype and *ace-1 dop-3* double mutants do not exacerbate the phenotype of the *ace-1* mutant. B.) *rab-3* mutants exhibit a moderate defect in egg-laying behavior and *tbc-4* mutants exhibit wild-type egg-laying behavior, but the phenotype of the *dop-3 tbc-4* double mutant demonstrates an additive increase in acetylcholine release. In addition, *rab-3; tbc-4* double mutants exhibit a minor egg-laying defect that demonstrates an antagonistic relationship between RAB-3 and TBC-4. Each bar represents the average egg count of 30 staged young adult animals dissolved in 20% hypochlorite solution. Bars represent the standard error of the mean. Asterisks represent the difference from wild-type by Student t test (*=$p<0.005$) and the statistical difference between two bracketed bars (** = $p<0.05$).

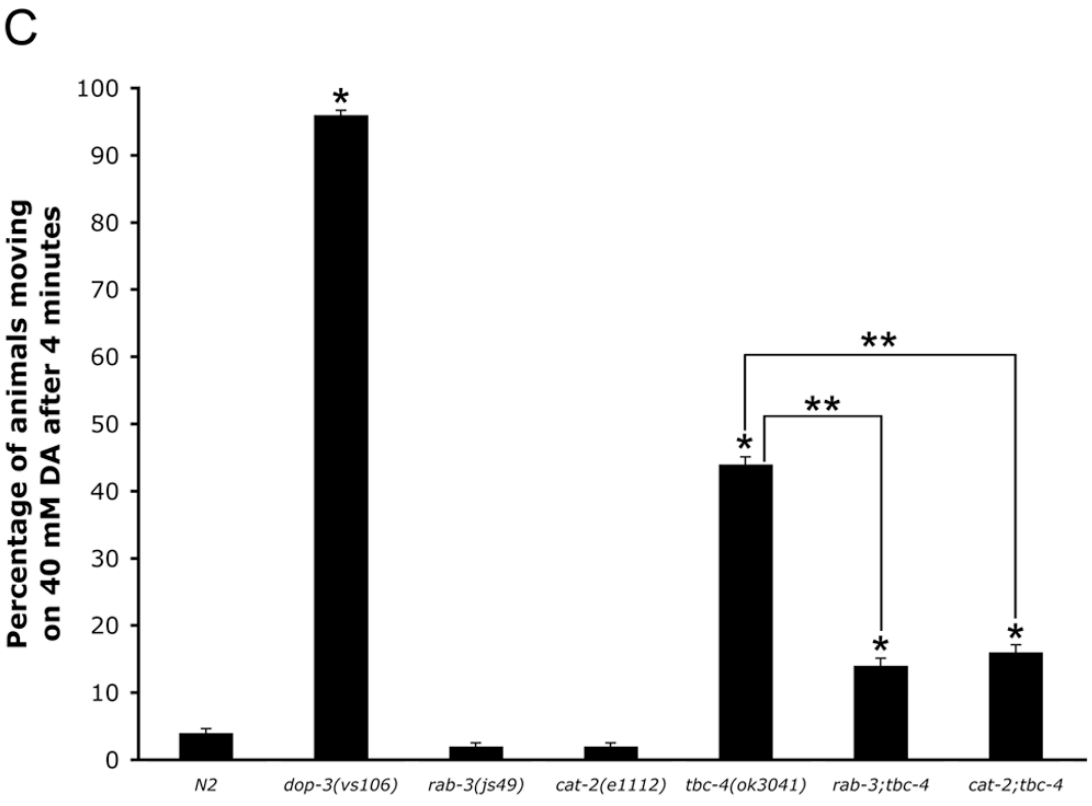
TBC-4 and DOP-3 both antagonize RAB-3- and AEX-6 –mediated acetylcholine release in response to exogenous dopamine

To further investigate the role of vesicular trafficking in dopaminergic regulation of acetylcholine release, we examined the response of mutants defective in presynaptic vesicular trafficking components to exogenous dopamine (Fig. 3.14). To begin this analysis, we first needed to demonstrate resistance to exogenous dopamine is conferred by altered cholinergic activity. Analysis of *ace-1* and *ace-2* mutants demonstrates that resistance to exogenous dopamine is in fact the result of increased cholinergic activity (Fig. 3.14*A*), and thus, resistance to exogenous dopamine can be used as a behavioral assessment of acetylcholine release. Subsequent analysis of mutations in vesicular trafficking components demonstrate that positive regulators of RAB-3-and AEX-6-mediated vesicular docking antagonize the dopamine resistance of both *dop-3* (Fig. 3.14*B*) and *tbc-4* (Fig. 3.14*C,D*) mutants, which supports the model of dopamine signaling modulating acetylcholine release through the regulation of vesicular docking.

DOP-3 selectively antagonizes RAB-3 in response to food

To investigate the role of vesicular docking in endogenous dopamine signaling, we chose to observe the ability of mutants in components of presynaptic vesicular docking to slow in response to food (Fig. 3.15). As a result of this analysis, we observed that mutations in the positive regulatory components of RAB-3-mediated vesicular docking cause a loss of the basal slowing response, and that these components appear to antagonize the effects of TBC-4 and DOP-3 (Fig. 3.15*A*).


A
Percentage of animals moving on 40 mM DA plates after 4 mintues
100
90
80
70
60
50
40
30
20
10
0
*
**
N2
dop-3(vs106)
ace-1(nd35)
ace-2(g72)
ace-2;ace-1(nd35)
B
Percentage of animals moving on 40 mM DA after 4 minutes
N2
dop-3 (vs106)
tbc-4 (ok3041)
rab-3 (js49)
rab-3;dop-3
aex-3 (js814)
aex-3 dop-3
rbf-1 (js232)
rbf-1; dop-3


C
Percentage of animals moving on 40 mM DA after 4 minutes
100
90
80
70
60
50
40
30
20
10
0
*
**
**
*
*
*
N2
dop-3(vs106)
rab-3(js49)
cat-2(e1112)
tbc-4(ok3041)
rab-3;tbc-4
cat-2;tbc-4

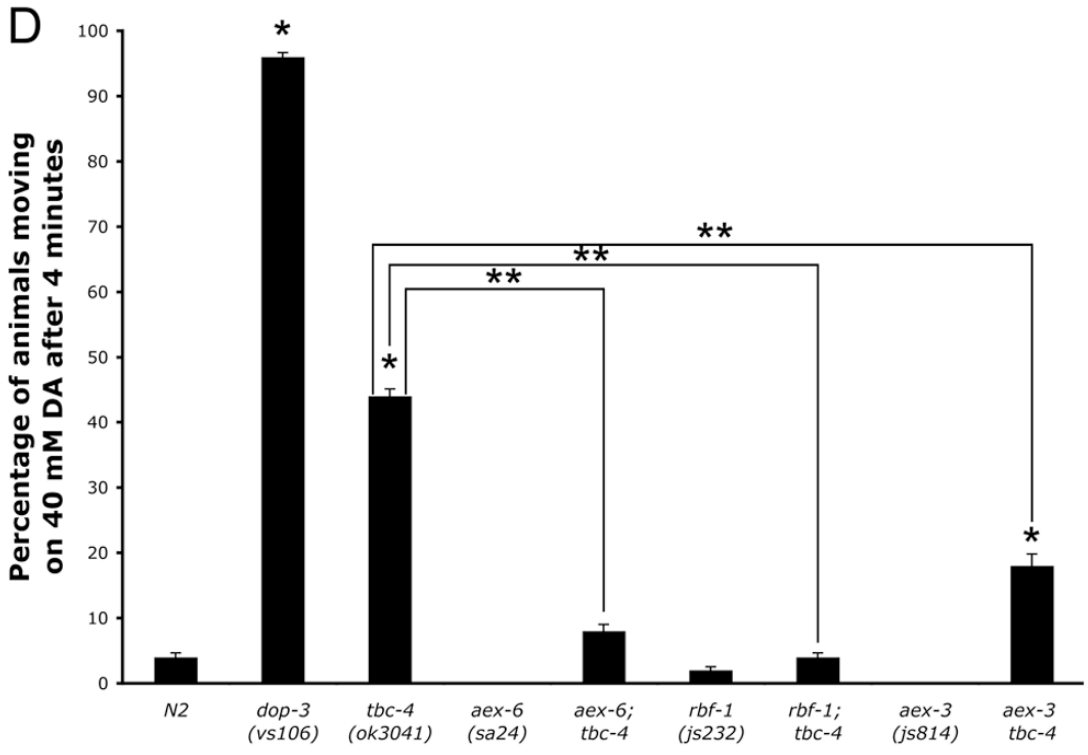

D
Percentage of animals moving on 40 mM DA after 4 minutes
100
90
80
70
60
50
40
30
20
10
0
*
**
**
**
*
*
N2
dop-3 (vs106)
tbc-4 (ok3041)
aex-6 (sa24)
aex-6; tbc-4
rbf-1 (js232)
rbf-1; tbc-4
aex-3 (js814)
aex-3 tbc-4

Figure 3.14. DOP-3 signaling is regulated by RAB-3- and AEX-6-mediated vesicular trafficking. A.) The dopamine resistance of *dop-3* mutants is due to increased acetylcholine release, as supported by the dopamine resistance of *ace-1*, *ace-2*, and *ace-1; ace-2* double mutants. B.) Double mutants between positive regulators of vesicle docking and *dop-3* reduce the dopamine resistance conferred by the *dop-3* mutation, demonstrating an antagonistic relationship between vesicular docking and DOP-3 receptor signaling. C.) The dopamine resistance of *tbc-4* is antagonized by RAB-3 and dependent on dopamine signaling as demonstrated by the diminished dopamine resistance of the *cat-2; tbc-4* double mutant. D.) The dopamine resistance of *tbc-4* is antagonized by mutations in positive regulatory components of vesicular docking. Bars represent the averages of 5 trials of 10 animals each for a total of 50 animals per strain. Error bars represent the standard error of the mean. Asterisks represent the statistical difference from wild-type (* = $p<0.001$) and the statistical difference between two bracketed bars (**=$p<0.005$).

In contrast to these findings, mutations in the positive regulatory elements of AEX-6-mediated vesicular docking do not cause a loss of the basal slowing response, and do not antagonize the effects of DOP-3 (Fig. 3.15*B*). Interestingly, although the *tbc-4(ok3041)* mutation has no effect on the phenotype of *rab-3* mutants, *aex-6; tbc-4* double mutants show a significant defect in basal slowing. These two observations support the model that RAB-3- but not AEX-6-mediated vesicular docking is required for the basal slowing response. In addition, we observed that mutations in AEX-6-mediated vesicular docking components disrupt the rate of locomotion of animals in the absence of food despite having no effect on the ability of the animal to slow in response to food, which when incorporated with our other observations suggests different roles for AEX-6 and RAB-3 in facilitating locomotion: RAB-3, like DOP-3, may only facilitate modulation of acetylcholine release under specific conditions such as slowing in response to food, whereas AEX-6 may play a broader role in mediating the overall locomotion rate of *C. elegans* independent of dopamine-specific regulation of acetylcholine release.

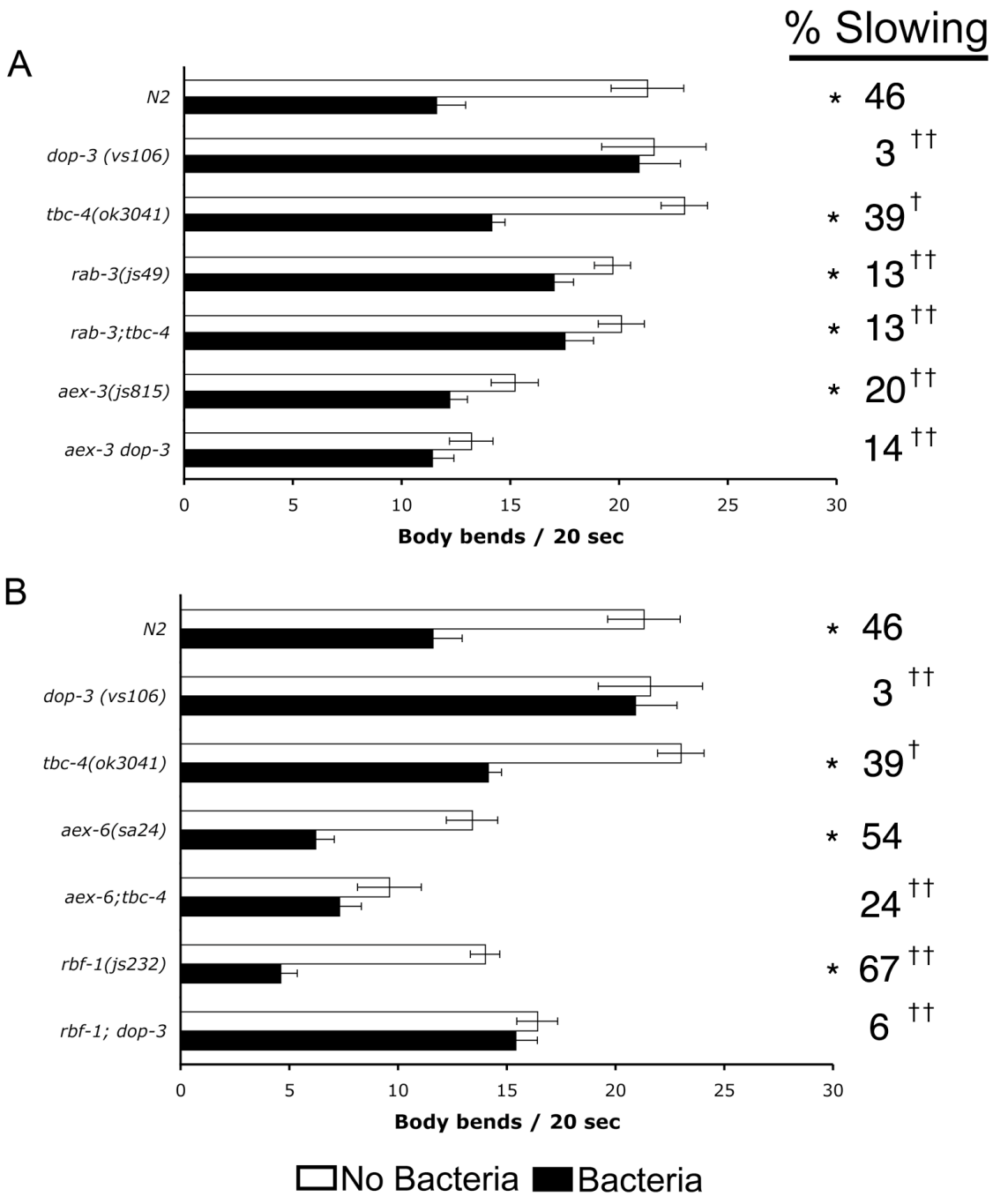


Figure 3.15. Mutants in dopamine signaling and vesicular trafficking components are defective in the basal slowing response. A.) *tbc-4* mutants have a minor defect in basal slowing and the *tbc-4(ok3041)* mutation does not alter the defects of *dop-3* or *rab-3* mutants, and mutants in positive regulatory components of RAB-3-mediated vesicular docking are defective in basal slowing and antagonize the basal slowing defect of *dop-3*. B.) The *tbc-4(ok3041)* mutation alters the wild-type basal slowing response of *aex-6* mutants, likely through a synthetic effect, and mutants in positive regulatory components of AEX-6-mediated vesicular docking are not defective in basal slowing and do not

antagonize the basal slowing defect of *dop-3*. L4 hermaphrodites were staged 14-19 hrs prior to the assay at 20º. Body bends were counted for five 20-second intervals for 6 young adult worms per strain for a total of 30 independent measurements per condition. White bars indicate locomotion rate in the absence of food, and black bars indicate locomotion rate in the presence of food. Error bars represent the 95% confidence interval. Asterisks indicate slowing based on a Student's t test ($p<0.005$).

Mutations in RAB-3 but not AEX-6 vesicular docking components rescue the SWIP phenotype of *dat-1*mutants.

As SWIP is a behavior mediated by dopamine signaling in the cholinergic motor neurons, we sought to observe the effects of mutations in vesicular docking components on the SWIP phenotype of *dat-1* mutants (Fig. 3.16). Analysis of these mutants demonstrates that the SWIP phenotype requires RAB-3 and TBC-4, but not AEX-6 or AEX-3. These observations are consistent with the model that RAB-3 function is required for the exhibition of specific dopamine-dependent behaviors, whereas AEX-6 is not. It is surprising, however, that the *aex-3(js814)* mutation has no effect on the SWIP phenotype of *dat-1* mutants, as AEX-3 has been shown to promote the activity of both RAB-3 and AEX-6 (Mahoney *et al.* 2006). It is also surprising that mutations in both *tbc-4* and *rab-3* rescue the SWIP phenotype of *dat-1* mutants, as this suggests that both RAB-3 and TBC-4 are required to inhibit acetylcholine release, an observation which is supported by the functional role of TBC-4 as a Rab GAP but counter-intuitive to the predicted function of a Rab GTPase. However, this observation is not unprecedented, as Rab3A has been demonstrated to inhibit neurotransmitter release under certain conditions in the mammalian nervous system (Geppert *et al.* 1997).

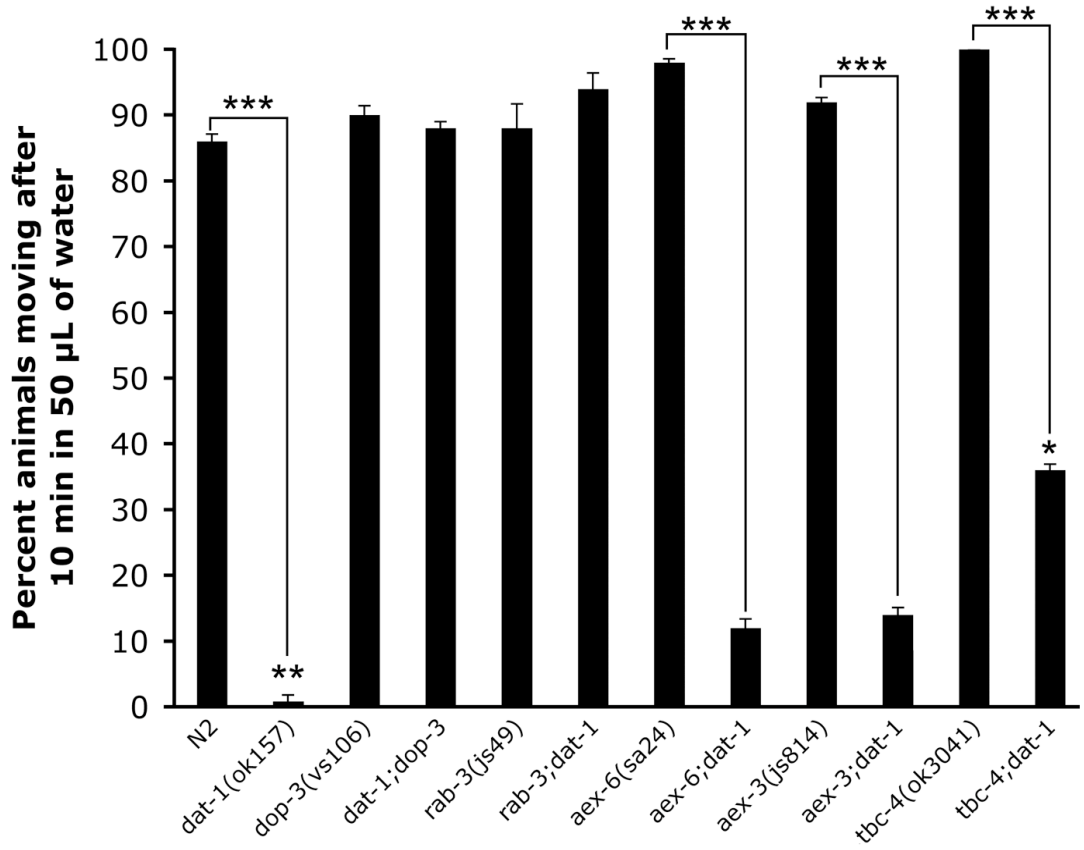


Figure 3.16. Mutations in *rab-3* and *tbc-4* rescue the SWIP phenotype of *dat-1* mutants. *rab-3* and *tbc-4* mutations rescue the SWIP phenotype of *dat-1* mutants, demonstrating that mutations in these genes increase acetylcholine release. In contrast, *aex-6* and *aex-3* mutations fail to rescue the SWIP phenotype of *dat-1* mutants, suggesting that mutations in these genes do not increase acetylcholine release. Bars represent the average of five trials of 10 L4 animals each for a total of 50 animals per strain. Error bars represent the 95% confidence interval. Asterisks represent statistical difference from N2 by Student t test (* = $p<0.005$, **=$p<0.001$) and the statistical difference between two bracketed bars (*** = $p<0.005$).

CHAPTER IV

DISCUSSION

Dopamine receptor signaling is a critical mechanism by which neurotransmission is regulated, and further investigation into the molecular pathways activated downstream of the receptors is crucial to achieve a better understanding of how the two classes of dopamine receptors interact to modulate neural activity. Here we provide evidence that dopamine acts through two antagonistic receptors to modulate acetylcholine release from the cholinergic motor neurons. We also characterize a novel component of dopamine signaling, the Rab GAP TBC-4, and demonstrate that vesicular docking mediated by the presynaptic Rabs RAB-3 and AEX-6 have distinct functional roles in facilitating locomotion in *C. elegans* despite their mutual regulation by TBC-4. Our results support a model of dopaminergic regulation of vesicular trafficking as one possible mechanism by which dopamine receptor signaling acts to modulate neural activity.

DOP-3 signaling inhibits acetylcholine release from the cholinergic motor neurons

D2-like receptors are characterized by their inhibitory effect on cAMP-dependent processes in the mammalian brain (Neve *et al.* 2004), and the *C. elegans* D2-like receptor DOP-3 has similarly been shown to inhibit neurotransmission (Chase *et al.* 2004). The expression pattern of DOP-3 in the ventral cord motor neurons in addition to its clear role in regulating locomotor activity suggests that signaling through the DOP-3 receptor inhibits acetylcholine release. Here we present several lines of evidence that demonstrate this hypothesis to be true. The isolation of both the *glr-1(nd38)* and *ace-1(nd35)* mutations from a genetic screen for mutants resistant to exogenous dopamine directly

correlates increased acetylcholine release with dopamine resistance. In addition, we demonstrate that the basal slowing response, a well-defined dopamine-dependent behavior, is facilitated through a DOP-3-mediated decrease in acetylcholine release. Furthermore, we establish SWIP as a dopamine-dependent behavior that is mediated by a DOP-3-mediated inhibition of acetylcholine release in the motor neurons of the ventral cord. Finally, we demonstrate through the use of acute aldicarb exposure not only that *dop-3* mutants exhibit increased acetylcholine release, but also that DOP-3 plays an active role in decreasing the release of acetylcholine from the hyper-activated cholinergic motor neurons of *glr-1* mutants. These observations firmly establish that DOP-3 receptor signaling inhibits acetylcholine release from the cholinergic motor neurons, a conclusion that supports mammalian studies of D2-like receptor inhibition of the tonically-active cholinergic interneurons of the striatum (Aosaki *et al.* 1994, Deng *et al.* 2007).

DOP-1 antagonizes DOP-3 signaling in the cholinergic motor neurons

While it is well established that D1- and D2-like dopamine receptors can exert either antagonistic or synergistic effects on neurotransmission (Neve *et al.* 2004), the underlying mechanism(s) for these interactions are largely unknown. Furthermore, it has been widely observed that the antagonism or synergism between these two receptor-signaling pathways occurs amongst different cells expressing only one class of receptor at detectable levels (Gerfen *et al.* 1990), although there is increasing evidence for D1- and D2-like receptor co-expression in specific cell types (Lee *et al.* 2004, Geldwert *et al.* 2006). Here we demonstrate that in *C. elegans*, DOP-1 antagonizes the inhibitory signaling of DOP-3 in the cholinergic motor neurons to modulate acetylcholine release

from these cells in the behaving worm. This is the first report to our knowledge of functional D1- and D2-like receptor antagonism within a single cell *in vivo*. This observation is an expected consequence of receptor co-expression and further supports evidence that D1- and D2-like receptors can function in a single cell to exert specific effects on neurotransmission (Lee *et al.* 2004). The variety of responses that can be elicited from interactions of D1- and D2-like receptors is impressive, and includes the formation of both homodimers and heterodimers (Lee *et al.* 2000) and synergistic or antagonistic receptor signaling between two cell types or within a single cell (Gerfen *et al.* 1990, Surmeier *et al.* 2007). This large array of signaling modalities is supported by the expression of roughly equal concentrations of heterogeneously expressed D1- and D2-like receptors in the medium spiny neurons of the striatum (Surmeier *et al.* 1996, Geldwert *et al.* 2006), thereby enabling the diverse mechanisms by which dopamine can regulate neurotransmission in these cells. The heterogeneous expression of D1- and D2-like receptors is conserved in *C. elegans*, as the sub-cellular localization of the DOP-1 and DOP-3 receptors supports this model (Kathryn Maher, unpublished data). Furthermore, we have shown that DOP-1 antagonism of DOP-3 provides a mechanism through which to modulate acetylcholine release, a finding that supports studies of the aspiny cholinergic interneurons of the striatum, where dopamine has been shown to alter the firing rate of these neurons to facilitate associative learning (Aosaki *et al.* 1994,Watanabe and Kimura 1998, Deng *et al.* 2007, Pisani *et al.* 2001).

TBC-4 is a Rab GAP in C. elegans with specificity for the presynaptic Rabs RAB-3 and AEX-6

As a result of our genetic screen to isolate mutants resistant to exogenous dopamine, we identified the putative Rab GAP TBC-4 as necessary component of exogenous dopamine response. Sequence analysis and comparison of the amino acid sequence of TBC-4 demonstrates that TBC-4 is indeed a Rab GAP, and that it closely resembles the mammalian Rab 27A-GAP EPI64. In mammalian cell culture, EPI64 regulates the activity of Rab 27A in melanocytes (Itoh and Fukuda 2006), but the *C. elegans* homolog of Rab 27A, AEX-6, is expressed presynaptically in the motor neurons of the ventral nerve cord along with the Rab 3A homolog RAB-3, where both Rabs facilitate neurotransmission (Nonet *et al.* 1997,Mahoney *et al.* 2006). Subsequent analysis of the expression pattern of TBC-4 revealed that TBC-4 was co-expressed in the cholinergic motor neurons with RAB-3, which suggested the possibility that these two proteins interact *in vivo*. Genetic analysis of the cholinergic inhibition of egg laying in *tbc-4* and *rab-3* mutants demonstrates that TBC-4 inhibits acetylcholine release and opposes RAB-3 facilitation of neurotransmission in the egg-laying circuit. These observations were further supported by a genetic analysis of dopamine resistance that revealed not only that dopamine resistance is caused by excess acetylcholine release, but also that both DOP-3 and TBC-4 antagonize RAB-3- and AEX-6-mediated vesicle trafficking through a dopamine-dependent mechanism. These results combined demonstrate that TBC-4 can exhibit GAP activity towards RAB-3 and AEX-6, and that this GAP activity is at least partially facilitated by dopamine signaling.

RAB-3 and AEX-6 facilitate synaptic transmission in distinctly different capacities

Behavioral analysis of dopamine-dependent TBC-4 regulation of cholinergic motor neuron activity revealed that while RAB-3 was required for dopamine-dependent inhibition of synaptic transmission, AEX-6 was not. Also, we found that while TBC-4 was required for dopamine-dependent inhibition of RAB-3, TBC-4 does not inhibit AEX-6 activity in response to dopamine. Therefore, AEX-6-mediated neurotransmission is not altered by the context-specific release of dopamine, whereas both TBC-4 and RAB-3 function is dramatically altered. These observations collectively support the model of RAB-3 and AEX-6 facilitating neurotransmission in separate and distinct capacities.

AEX-6 facilitates acetylcholine release from the cholinergic motor neurons (Mahoney *et al.* 2006) and its activity is not altered by dopamine signaling (our results). However, the locomotion rate of *aex-6* mutants is significantly slower than that of wild-type animals (Thomas 1990), which is not true of *rab-3* mutants (Nonet *et al.* 1997, our observations). These observations together suggest that AEX-6 acts to tonically regulate the output of the cholinergic motor neurons independent of context-specific stimuli (Fig. 4.1) as a possible component of the *C. elegans* central pattern generator which has been previously observed to require synchronization of both GABAergic and cholinergic output (Karbowski *et al.* 2008), or that AEX-6 activity may be regulated by additional context-specific cues (Fig. 4.1*B*).

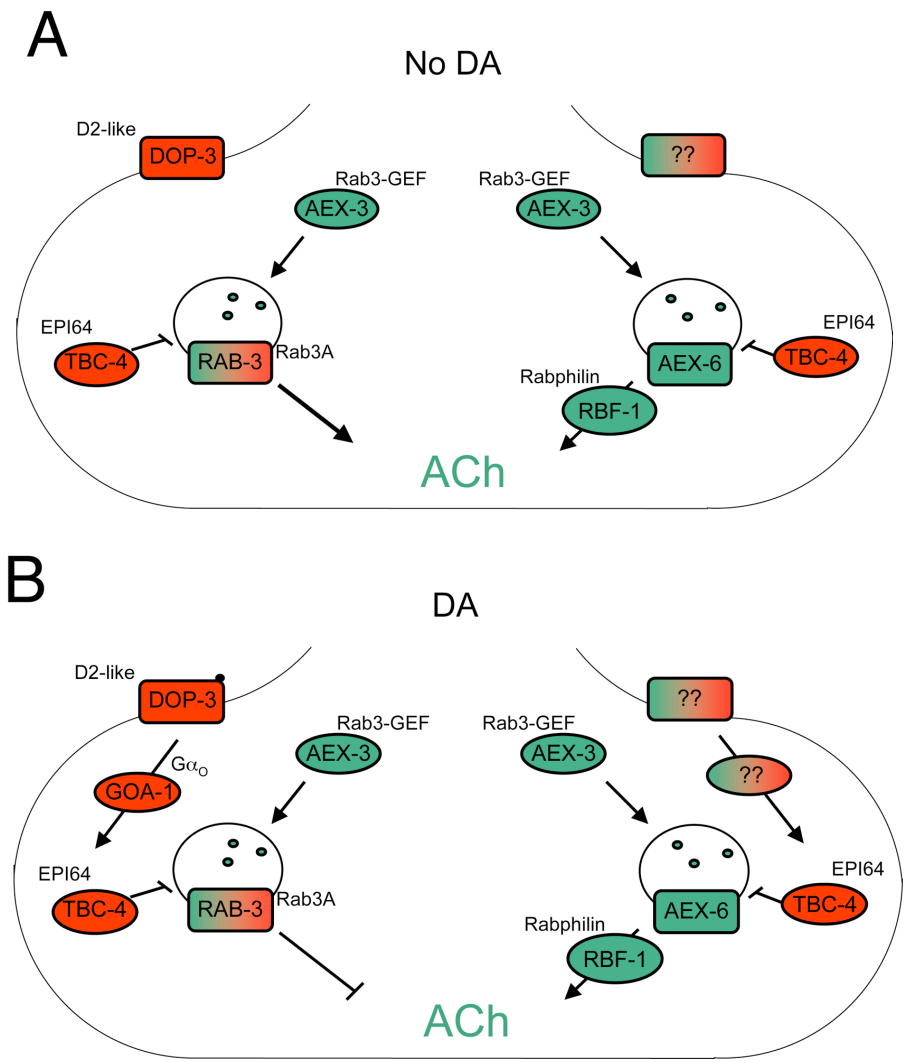


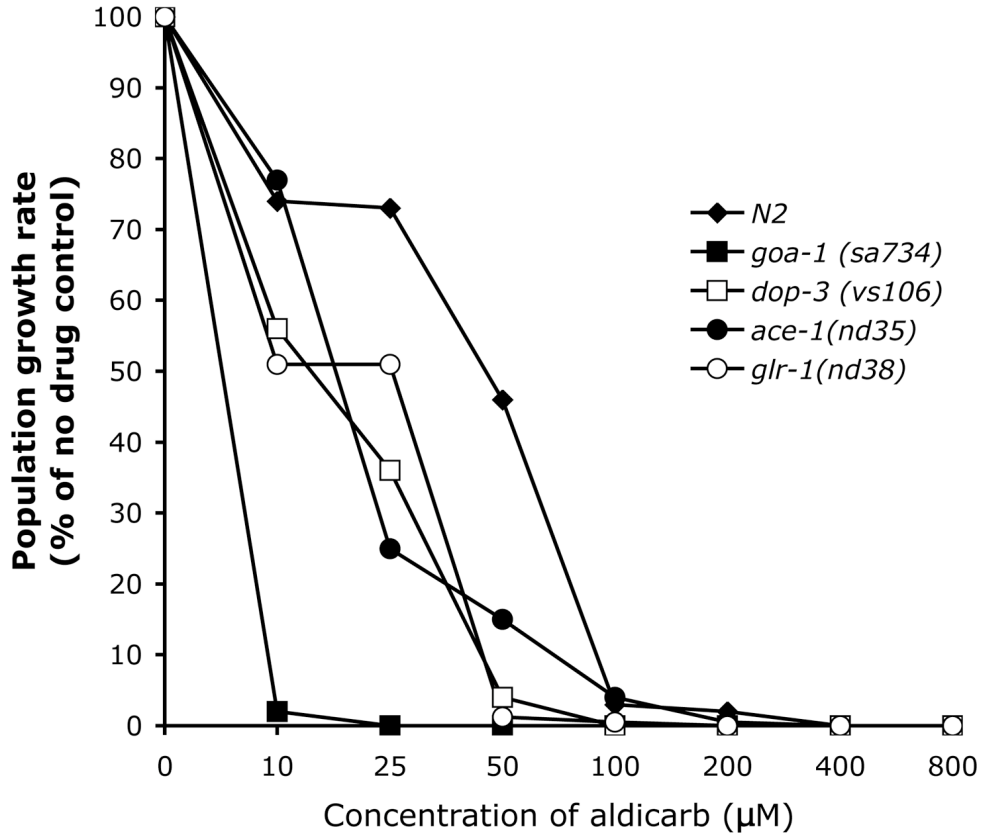
Figure 4.1. Dopamine signaling through DOP-3 may limit the quantal release of acetylcholine through a RAB-3-mediated mechanism. A.) In the absence of dopamine signaling through DOP-3, RAB-3 mediates the docking of loaded vesicles to the active zone of the presynaptic terminal and AEX-6 facilitates the association of loaded vesicles with the SNAP-NSF complex (Nonet *et al.* 1997, Mahoney *et al.* 2006). B.) RAB-3 inhibits acetylcholine release in response to dopamine signaling through DOP-3, whereas AEX-6 signaling is unaffected.

RAB-3 appears to inhibit acetylcholine release in response to dopamine, which is counter-intuitive to the well-established role of Rab proteins as facilitators of vesicle docking and fusion (Zerial and McBride 2001), and is also contrary to the pre-established role of RAB-3 in *C. elegans* as a facilitator of active-zone recruitment of synaptic vesicles (Nonet *et al.* 1997). However, Rab3A has been shown to facilitate

neurotransmission through two separate mechanisms. One of these mechanisms is the recently well-established and highly-conserved recruitment of synaptic vesicles to the presynapse through the formation of a tri-partite complex with Munc13 and Rim1a (Weimer *et al.* 2006, Gracheva 2008, Dulubova *et al.* 2005) which increases the amount of docked vesicles at the active zone ready for release. The other mechanism is the limitation of quantal release of neurotransmitter at the active zone by a distortion of the plasma membrane (Geppert *et al.* 1997, Wang *et al.* 2008). This mode of regulation is unique to Rab3A and supports our observations of an inhibitory role of RAB-3 in *C elegans* neurotransmission. Furthermore, our results support a context-specific "switch" that reverses RAB-3 facilitation of neurotransmission in response to dopamine, possibly through the activation of TBC-4 (Fig. 4.1*B*). This context-specificity may help to explain the paradoxical role of Rab3A in neurotransmission in the mammalian brain and more clearly define the role of Rab proteins in facilitating calcium-triggered quantal release from presynaptic terminals in the central nervous system.

APPENDIX

MUTANTS RESISTANT TO EXOGENOUS DOPAMINE BUT INSENSITIVE TO HIGH DOSES OF ALDICARB DISPLAY ALDICARB SENSITIVITY AFTER CHRONIC EXPOSURE TO LOW DOSES



dop-3 and *ace-1* mutants are not sensitive to high concentrations of aldicarb, despite direct evidence that these mutants have increased levels of acetylcholine release. However, both *ace-1* and *dop-3* mutants are sensitive to chronic exposure of low levels of aldicarb, as is the *glr-1* mutant identified in the screen, consistent with the hypothesis that mutations in inhibitory components of dopamine signaling result in increased acetylcholine release. For each experiment, enough L4 animals were staged to lay approximately 300 progeny 96 hours later on plates without aldicarb. Experiments for each strain were done in triplicate, and results are plotted as the percent population present on the no drug control plates.

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