aex-3 Encodes a Novel Regulator of Presynaptic Activity in C. elegans

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Summary

C. elegans aex-3 mutations cause pleiotropic behavioral defects that are suggestive of reduced synaptic transmission. aex-3 mutations also show strong genetic interactions with mutations in unc-31 and unc-64, two other genes implicated in synaptic transmission. Physiological and pharmacological studies indicate that aex-3 defects are presynaptic. In aex-3 mutants, the synaptic vesicle-associated RAB-3 protein aberrantly accumulates in neuronal cell bodies and is reduced in synapse-rich axons. This localization defect is specific to RAB-3, since other synaptic proteins are localized normally in aex-3 mutants. aex-3 encodes a 1409 amino acid protein with strong homology to DENN, a human protein of unknown function. In C. elegans, aex-3 is expressed in all or nearly all neurons. These results suggest that AEX-3 is a novel regulator of presynaptic activity that interacts with RAB-3 to regulate synaptic vesicle release.

Introduction

Synaptic vesicle release at presynaptic terminals is a highly regulated process that is important for communication between neurons and at the neuromuscular junction. Several steps appear to be important for the process (reviewed by Südhof, 1995). First, synaptic vesicles containing neurotransmitter accumulate at specialized regions of the presynaptic membrane. Once there, docking and priming processes are thought to be required to prepare these vesicles for the rapid exocytosis that is characteristic of excitable cells. Finally, Ca$^{2+}$ influx stimulates the exocytosis event. A variety of proteins have been implicated in this process. Of specific interest for this paper are members of the small GTP-binding family called rab proteins. Various members of the rab family have been shown to regulate vesicular transport steps in neuronal and nonneuronal cells (reviewed by Ferro-Novick and Novick, 1993). A subgroup of the rab family, the rab3-related proteins, appear to be specifically important in secretory events in excitable cells. Rab proteins alternate between a GTP-bound state that is associated with vesicles and a GDP-bound state that is not associated with vesicles (Fischer von Mollard et al., 1991; Fischer von Mollard et al., 1994a; Stahl et al., 1994). These two states are thought to regulate vesicle exocytosis, though the precise role of rab proteins remains unclear.

Many of the proteins that regulate neuronal synaptic vesicle release have been identified by biochemical methods. These methods of identification typically require either direct interaction between these proteins and synaptic vesicles or some prior knowledge of the biochemical function of the proteins. In addition, biochemical analysis has limited potential for testing whether a protein is required for synaptic function in vivo. The utility of a genetic approach to the regulation of secretion is demonstrated by the remarkable success of such analysis in yeast (Ferro-Novick and Novick, 1993). For example, the key role of rab proteins in specific steps of vesicle transport in vivo has been demonstrated by analysis of temperature-sensitive mutations of the rab-encoding genes sec4 and ypt1 (Salimen and Novick, 1987; Segev et al., 1988).

C. elegans is well suited to a genetic analysis of neuronal synaptic transmission. C. elegans has a simple nervous system of 302 neurons in the hermaphrodite. Genetic analysis is facilitated because mutants with severely perturbed general neuronal function are often viable. Nevertheless, C. elegans neurons regulate a wide range of behaviors and appear in many ways to function similarly to vertebrate neurons. Specifically, several steps in the mechanism of vertebrate synaptic vesicle function are known to be conserved in nematodes. Synaptic vesicles are transported to presynaptic sites by a kinesin-like protein (Hall and Hedgecock, 1991; Okada et al., 1995). Many vertebrate neurotransmitters are found in C. elegans, and these are synthesized and loaded into vesicles by similar proteins (Alfonso et al., 1993, 1994; McIntire et al., 1993; Y. Jin, personal communication). Finally, synaptic vesicle exocytosis is regulated by nematode homologs of synaptotagmin (Nonet et al., 1993), Munc-18 (Gengyo-Ando et al., 1993; Hata et al., 1993), Munc-13 (Maruyama and Brenner, 1991; Brose et al., 1993), p145-CAPS (Livingstone, 1991; Wal lent et al., 1992), and rab3 (Nonet et al., unpublished data).

We and others previously identified aex-3 mutants based on specific behavioral defects (Thomas 1990; Avery 1993). Here, we report that aex-3 mutants have a variety of behavioral, physiological, and pharmacological abnormalities that are consistent with a generalized defect in presynaptic activity. We present evidence that aex-3 encodes a predicted protein of 1409 amino acids with strong similarity to a human protein of unknown function called DENN. These results suggest that aex-3 encodes the defining member of a protein family not previously implicated in synaptic vesicle release.

Results

aex-3 Mutants Have Pleiotropic Behavioral Defects

aex-3 mutants were first isolated in a screen for altered defecation behavior (Thomas, 1990). aex-3 alleles have
also been recovered in screens for feeding defects (Avery, 1993) and aldicarb resistance (Miller et al., 1996; Nonet et al., unpublished data; E. Jorgensen, personal communication). We find that the two most prominent defects in aex-3 mutants are an abnormal defecation motor program and poor male mating efficiency. The C. elegans defecation motor program consists of a stereotyped series of three muscle contractions (Thomas 1990). First, posterior body-wall muscles contract (pBoc) and then relax, causing gut contents to accumulate near the anus. Three to four seconds later, anterior body-wall muscles contract (aBoc) to pressurize the gut contents. Finally, specialized enteric muscles contract to expel the gut contents from the anus (Exp). In all five aex-3 mutants analyzed (ad418, sa5, ad696, n2166, and y255), pBoc appeared normal, but only 10%-20% of defecation motor program cycles had a visible aBoc or Exp (hence the gene name aex). In a quantitative mating assay, aex-3 males sired far fewer cross progeny than wild-type males (see Experimental Procedures). Direct observation showed that aex-3 mutant males displayed mating behavior less frequently when they encountered hermaphrodites (data not shown). In addition to these severe defects, aex-3 mutants had other mild behavioral defects. aex-3(ad696) and aex-3(sa5) mutants retained slightly more eggs than the wild type, indicative of a mild egg-laying defect. Finally, aex-3 mutants intermittently displayed exaggerated sinusoidal bends of the body during locomotion, a phenotype that was most pronounced in aex-3(y255). No obvious aex-3 allelic series could be constructed based on the severity of the various behavioral defects. The range of defects observed in aex-3 mutants suggests a general nervous system abnormality.

**aex-3 Has Strong Genetic Interactions with Other Synaptic Mutants**

Additional evidence suggesting a synaptic function for aex-3 comes from genetic interactions affecting dauer formation. The dauer is an alternative larval form that is induced in response to harsh environmental conditions (reviewed by Thomas, 1993). The dauer larva can survive for several months without feeding before resuming development. Dauer formation is controlled by a neuronal circuit, and unregulated dauer formation can result from defective nervous system function.

In the course of genetic analysis of aex-3, we found that double mutants between aex-3 and either unc-31 or unc-64 displayed strong dauer formation—constitutive (Daf-c) phenotypes. unc-31 encodes a nematode homolog of p145, a calcium-dependent activator of secretion (Livingstone 1991; Walent et al., 1992), and unc-64 encodes a homolog of syntaxin (M. Nonet, unpublished data). Both of these proteins are implicated in synaptic vesicle release. Whereas none of the single mutants had an obvious Daf-c phenotype, double mutants in all possible combinations among aex-3, unc-31, and unc-64 alleles displayed a strong Daf-c phenotype (Figure 1). These results suggest that aex-3, unc-31, and unc-64 function together in related processes.

### Electropharyngeogram Indicates Synaptic Transmission Defects in aex-3 Mutants

To test more directly for synaptic transmission defects in aex-3 mutants, we examined activity in the pharyngeal nervous system of these animals using an extracellular recording technique developed by Raizen and Avery (1994). This technique allows for visualization of currents intrinsic to muscle action potentials as well as those resulting from synaptic input (Figure 2). Currents elicited by two distinct motor neurons can be identified. First, attempts by MC to trigger a muscle action potential. Whereas none of the single mutants had an obvious Daf-c phenotype, double mutants in all possible combinations among aex-3, unc-31, and unc-64 alleles displayed a strong Daf-c phenotype (Figure 1). These results suggest that aex-3, unc-31, and unc-64 function together in related processes.

### Pharmacological Analysis of aex-3 Mutants Suggests a Defect in Presynaptic Function

To test further whether aex-3 mutants have defects in synaptic transmission, we measured sensitivity to the

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Figure 1. Synthetic Dauer Formation Caused by unc-31, unc-64, and aex-3 Mutations

Constitutive dauer formation in single and double mutants of unc-31(u280), unc-64(e246), and aex-3(y255) alleles. For each genotype, three assays were performed at 25°C, and the mean is shown. N is the total number of animals assayed for each genotype, and the bars show the SEM. All possible combinations among unc-64(e246), unc-64(md130), unc-31(e169), unc-31(e928), unc-31(u280), aex-3(ad418), aex-3(sa5), aex-3(ad696), aex-3(n2166), and aex-3(y255) were tested, and results were consistent in every case.
acetylcholinesterase inhibitor aldicarb. Resistance to aldicarb results from reduced presynaptic acetylcholine release and is well correlated with defects in synaptic transmission (Nonet et al., 1993; Miller et al., 1996). We found that all aex-3 mutants resisted paralysis induced by treatment with 0.5 mM of aldicarb for 6 hr (Figure 3A). As a positive control, we tested a mutant of lev-1, which encodes a subunit of a nicotinic acetylcholine receptor (Fleming et al., 1993). As expected, the lev-1 mutant strongly resisted aldicarb in our assay (Figure 3A). The aldicarb resistance of aex-3 mutants could thus be due to either pre- or postsynaptic defects.

To determine whether the aex-3 defect is pre- or postsynaptic, we measured sensitivity of aex-3 mutants to levamisole, an acetylcholine receptor agonist in nematodes (Lewis et al., 1980). We found no significant difference in sensitivity to levamisole (20, 60, and 100 μM) between an aex-3(n2166) mutant and the wild type (Figure 3B; data not shown). We also tested other aex-3 alleles and found similar levamisole sensitivity (data not shown). As expected, the lev-1 mutant strongly resisted levamisole (Figure 3B), consistent with its postsynaptic defect. These pharmacological assays indicate that aex-3 mutants are impaired in presynaptic acetylcholine release. Together with the pleiotropic behavioral defects, these results suggest that aex-3 mutants confer a general defect in presynaptic activity.

**RAB-3 Protein Mislocalizes in aex-3 Mutants**

In an attempt to determine how aex-3 mutations impair presynaptic activity, we examined aex-3 mutants using antibodies specific for two synaptic vesicle components, SNT-1 (synaptotagmin; Nonet et al., 1993) and RAB-3 (a rab3 homolog, Nonet et al., unpublished data). In the wild-type and in the aex-3(y255) mutant, anti-SNT-1 antiserum stained only the synapse-rich axons and not cell bodies (Figure 4), suggesting that SNT-1 is correctly localized to synaptic terminals in the aex-3 mutant. Anti-RAB-3 antisera stained predominantly axons in wild-type animals, though faint staining of occasional cell bodies was visible (Figure 4). In contrast, in the aex-3 mutant, anti-RAB-3 antisera stained predominantly cell bodies (Figure 4). This observation suggests that aex-3 is required for proper localization of RAB-3 protein in axons and confirms a general presynaptic defect.

**Molecular Analysis of aex-3**

We used a positional cloning approach to determine the molecular identity of aex-3. Genetic map data (see Experimental Procedures) indicated that aex-3 lies approximately midway between egl-17 and unc-1 near the left tip of the X chromosome (Figure 5A). Based on a correlation of the physical and genetic maps from this region, we tested several cosmids for transgenic rescue of the Aex phenotype (Mello et al., 1991). Cosmid C02H7 rescued the Aex phenotype, but two overlapping cosmids, M02A10 and R04F4, did not rescue (Figure 5A). To further narrow the potential aex-3 region, three λ phage subclones and two plasmid subclones derived from cosmid C02H7 were tested for transgenic rescue (Figure 5A).

A Northern blot probed with the insert from the smallest rescuing clone (pTJ 500) revealed a single candidate aex-3 mRNA of about 5 kb (Figure 5B). Using the same probe, one cDNA clone was isolated from ~300,000 plaques from a cDNA library (Barstead et al., 1991). This cDNA had a 1.7 kb insert and a poly-(A) stretch at one end, suggesting that the insert corresponds to the 3' end of the aex-3 mRNA. During this study, the C. elegans Genome Sequencing Consortium completed this region of the X chromosome (Wilson et al., 1994; C. elegans Genome Sequencing Consortium, personal communication). Gene finder analysis of the aex-3 rescuing region predicted a single gene product (P. Green, personal communication).
Figure 3. Pharmacological Assays
(A) Aldicarb sensitivities of aex-3 mutants and the wild type. Aldicarb sensitivities of aex-3(n2166) and wild-type animals were first determined at several different doses and times of exposure (data not shown). Based on these assays, a 6 hr exposure to 0.5 mM aldicarb was selected because this test readily distinguished wild-type from mutants sensitivities. The genotype of each strain is shown under each bar. Twenty animals per plate were tested (3 plates for the others). At the top of each bar, the SEM is shown. (B) Levamisole sensitivities of an aex-3 mutant and the wild type. The x-axis shows time (minutes) after exposing animals to 60 μM levamisole. The y-axis shows the percentage of paralyzed animals. Twenty animals per plate were tested (three plates per strain).

Discussion

aex-3 Mutants Have a Presynaptic Defect
Various experimental observations indicate that AEX-3 functions presynaptically to regulate transmitter release in many classes of neurons. aex-3 mutants are abnormal in a wide variety of behaviors, suggesting that the defects are not limited to a single neurotransmitter system. Additionally, defects in excitatory MC (probably cholinergic; Raizen et al., 1995) and inhibitory glutamatergic M3 synaptic transmission are observed in extracellular recordings of pharyngeal activity. While neither the behavioral defects nor the pharyngeal recordings address whether the defect in transmission in aex-3 mutants is pre- or postsynaptic, several other lines of evidence support a presynaptic function for AEX-3. First, the combined sensitivities of aex-3 mutants to a cholinergic receptor agonist and to an acetylcholinesterase inhibitor support a presynaptic defect. Second, the fact that expression of aex-3 is limited to neurons suggests a presynaptic site of action at neuromuscular junctions. Third,
Presynaptic Defects in aex-3 Mutants

Figure 4. Localization of SNT-1 and RAB-3 in aex-3 Mutants
Wild-type (A) and aex-3(y255) (B) animals stained with anti-synaptotagmin antisera. In both wild-type and aex-3 mutants, synaptotagmin immunoreactivity was restricted primarily to the ventral cord where many neuromuscular synapses are found. DAPI staining of the wild type (C), the same animal as [A] and aex-3 mutant (D), the same animal as [F] shows cell bodies. Wild type (E) and aex-3(y255) (F) animals stained with anti-RAB-3 antiserum. Specificity of the antiserum was demonstrated by absence of staining in rab-3 mutants. In wild-type animals, immunoreactivity is restricted primarily to the ventral cord. In contrast, in aex-3 animals, much of the RAB-3 immunoreactivity is located in neuronal cell bodies. In (D) and (F), closed arrows indicate examples of cells showing this pattern. Similar staining was observed for sa5 and n2166 (data not shown).

the mislocalization of the synaptic vesicle-associated protein RAB-3 in aex-3 mutants indicates a presynaptic function for AEX-3. Thus, behavioral, physiological, pharmacological, molecular, and cellular evidence suggests that AEX-3 is a panneuronal regulator of synaptic transmission.

Genetic Interactions among aex-3, unc-31, and unc-64
All double-mutant combinations of aex-3, unc-31, and unc-64 displayed a synthetic Daf-c phenotype, suggesting that these genes function in regulating dauer formation. Identified sensory neurons are known to regulate dauer formation (Bargmann and Horvitz, 1991; Schackwitz et al., 1996), but little is known about synaptic events in this process. Our results suggest that certain defects in synaptic transmission can result in a Daf-c phenotype. We hypothesize that aex-3, unc-31, and unc-64 each function in different aspects of synaptic vesicle exocytosis. Mutations in each gene may impair synaptic function to a degree that is insufficient to induce dauer formation. However, combinations of these mutations may reduce synaptic function below some threshold, resulting in a strong Daf-c phenotype.

AEX-3 May Regulate RAB-3 Function
The specific failure of RAB-3 protein to localize to synaptic regions in aex-3 mutants suggests that aex-3 in some way regulates rab-3 function. Consistent with this, the electrophysiological defects of rab-3 and aex-3 mutants are very similar (Nonet et al., unpublished data). However, behavioral defects point to roles for aex-3 beyond regulating rab-3 activity. aex-3 mutants have abnormal defecation motor steps and slight egg-laying defects that are not observed in the rab-3 null mutant (Nonet et al., unpublished data). In addition, male mating is more severely affected in aex-3 mutants. One possible explanation for these behavioral differences is that mislocalization of RAB-3 in aex-3 mutants causes abnormalities beyond those resulting from the loss of RAB-3 function. A more likely possibility is that aex-3 functions to regulate other proteins in addition to RAB-3. One intriguing possibility is that aex-3 is required for the proper localization of other rab proteins associated with synaptic vesicles such as rab5 (deHoop et al., 1994; Fischer von Mollard et al., 1994b).

Mislocalization of RAB-3
Furthering our understanding of AEX-3 will require determining how aex-3 mutations cause mislocalization of RAB-3 protein. We consider three likely possibilities. One possibility is that geranylgeranyl transferase (GGT) activity is impaired in aex-3 mutants. Rab3 is known to be geranylgeranylated at the C-terminus, and this modification is important for rab3 attachment to synaptic vesicles (Johnston et al., 1991). GGT consists of two subunits, components A and B (Seabra et al., 1992; Andres et al., 1993). Component B has GGT activity,
and component A is thought to present rab3 protein to component B (Andres et al., 1993). AEX-3 has no homology to component A or B and therefore is unlikely to encode these functions in C. elegans. It remains possible that aex-3 is less directly required for this process.

A second possibility is that RAB-3 guanine-nucleotide exchange activity is impaired in the aex-3 mutant. Exchange of GDP for GTP on rab3 is thought to activate transport to synaptic vesicles (Stahl et al., 1994). Guanine-nucleotide exchange on rab3 is promoted by MSS4 in mammals (Burton et al., 1994). AEX-3 has no homology to MSS4 or other guanine-nucleotide exchange factors, but it might form a novel class of nucleotide exchange factor, or it might be required indirectly for this process.

A third possibility is that AEX-3 is a RAB-3 binding protein of novel but unknown function. A variety of rab3 binding proteins have been identified and sequenced. These include rabphilin3a (Shirataki et al., 1993), rabin3 (Brondyk et al., 1995), and GDI (Sasaki et al., 1990). AEX-3 has no homology to any of these rab3 binding proteins, but it might encode a novel RAB-3 binding protein that promotes transport of RAB-3 to presynaptic terminals or binding of RAB-3 to synaptic vesicles.

Our work has identified a novel synaptic regulator that impairs synaptic transmission, probably in part by affecting RAB-3 function. Two roles for rab3 protein have been previously suggested: 1) rab3 acts as a negative regulator of vesicle release (Oberhauser et al., 1992; Johannes et al., 1994; Olszewski et al., 1994), or 2) rab3 acts to enhance vesicle release during repetitive stimulation (Geppert et al., 1994a). In C. elegans rab3 and aex-3 mutants, the strength and synchrony of M3 synaptic transmission to pharyngeal muscle is clearly reduced, not increased. Furthermore, aex-3 mutations act synergistically with other mutations that decrease synaptic transmission. Thus, our experiments are more consistent with roles for RAB-3 and AEX-3 in promoting rather than inhibiting synaptic vesicle release.

Experimental Procedures

Genetic Mapping

Methods for C. elegans culture, genetic analysis (Brenner 1974; Sulston and Hodgkin 1988), and nomenclature (Hornitz et al., 1979) were as previously described. The following mutations were used: LGIII unc-64(e246, md130) LGIV unc-31(e169, e928, u280)
Presynaptic Defects in aex-3 Mutants

Figure 6. Sequence of the Predicted AEX-3 Protein Amino Acid Number (Left)
The entire protein is 1409 amino acids long. The underlined sequences are the domains with strong homology to DENN. A BLASTP search of the "nonredundant" database in September 1996 revealed a single highly significant hit (P = 2.0; e-178). The identity between AEX-3 and DENN is 50.7% in the first domain, 31.7% in the second domain, and 43.2% in the third domain, using the BESTFIT program. One part of the first domain also has weak similarity to a HeLa tumor suppressor gene (HTS-1) (Lichy et al., 1992). The boxed sequence is a proline-rich domain. Lys 989, which is mutated to Arg in ad696, is indicated by a triangle. On the right is an alignment of AEX-3 and DENN generated by Clustal W; black boxes indicate amino acid identities, and gray boxes indicate conservative changes.

LGV dpy-11(e224)

LGO egl-17(e1313), aex-3(ad418), sa5, ad696, n2166, y255, unc-1(e580), lin-15(n765ts)

Construction of Double Mutants
For unc-31; aex-3 double mutants, unc-31/− males were mated with aex-3 hermaphrodites. From doubly heterozygous F1 hermaphrodites, F2 Aex progeny were picked and scored for segregation of the Aex phenotype. Four of nine recombinants were egl-17 unc-1/eql-17 and the other five recombinants were egl-17 unc-1/eql-17 aex-3.

Pharmacological Assays
J ust before pouring 35 mm plates, aldicarb (100 mM in 70% ethanol) or levamisole (100 mM in water) was added to NG agar at the appropriate concentration. After seeding with bacteria, 20 young adult progeny were picked. From confirmed unc-64 homozygotes, Unc-31 segregants could be distinguished based on their more severe Unc and Egl phenotypes. The resulting unc-31; unc-64 strains were crossed with N2 males to confirm segregation of the two different Unc phenotypes.

Behavioral Assays
Defecation assays were performed as described (Liu and Thomas, 1992). Twenty adult animals grown at 15°C were allowed to lay eggs for 3-5 hr at room temperature. After removing the adults, the plates were placed at 25°C. ~2 days later, dauers and nondauers were counted. Tests showed that recovery from the dauer stage in these strains is very poor at 25°C. Because double mutants containing unc-64(md130) grew asynchronously, these strains were observed several times over 24 hr, and dauers and L3 larvae were counted and removed at each time.

Microinjection Rescue
Microinjection was carried out as described (Mello et al., 1991). Cosmid or plasmid DNAs were purified using Qiagen columns following the manufacturer's protocol. pRF4 (rol-6 marker plasmid)
detected negative peaks with an amplitude of the peak of the R-phase transient (Raizen and Avery, 1994). M3 RACE system was used. In each case, Southern blot hybridization was defined as the time from the peak of the E-phase transient to aex-3.

Molecular biological methods were essentially as described (Sam-OLG289-GGAATTCGTCTTTTTGTGGTGAGCATC

Nucleic Acid Analysis were identified as transients occurring in the absence of a pump. with primers OLG292±OLG293 and OLG294±OLG295 was per-

ground occurring within 200 ms preceding a pump. We defined introduced restriction sites (either SalI, EcoRI, or BamHI), which

phage library (Stratagene) probing with both 2.0 and 2.2 kb EcoRI OLG291-GGAATTCGGGTTCTGGTATGTTCACTG

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lin-15(n765ts) animals were injected with pTJ500 (pbHL98 (lin-15 marker) and test DNA were coinjected into

non-Lin progeny from injected animals, the progeny were grown at 418 of AEX-3 (pTJ671). Similarly, pTJ500 was digested with SpeI, and test DNA were coinjected into

m

l, and 20 ng/μl, respectively. To find non-Lin progeny from injected animals, the progeny were grown at 25°C.

Immunocytochemistry was performed as described (Nonet et al., 1993), except that worms were fixed in a modified Bowin’s fixative (0.75 ml saturated picric acid, 0.25 ml formalin, 0.05 ml glacial acetic acid, 0.25 ml methanol, and 0.01 ml B-mercaptoethanol). Antisera against SNT-1 and RAB-3 are described elsewhere (Nonet et al., 1993, and unpublished data).

Electrophysiology Electropharyngeograms were recorded using an AC preamplifier (designed by David Brumley, University of Oregon) and LabView Acquisition software as previously described (Avery et al., 1995). The bath solution consisted of M9 with occasional addition of 2.5 mM serotonin to stimulate pumping. Young adult hemaphrodites were used for all analysis. Only records with at least 10 pharyngeal pumps were used in analysis. The duration of the pharyngeal pumps was defined as the time from the peak of the E-phase transient to the peak of the R-phase transient (Raizen and Avery, 1994). M3 transients were identified using a peak detection algorithm, which detected negative peaks with an amplitude of ~10% of the mean amplitude of the R-phase transients in a record. This algorithm identified ~99% of M3 transients visible in wild-type records. MC activity was scored as spikes distinguishable by eye from background occurring within 200 ms preceding a pump. We defined bursts as multiple transients occurring in this interval. MC failures were identified as transients occurring in the absence of a pump.

Nucleic Acid Analysis Molecular biological methods were essentially as described (Sambrook et al., 1989). PH14 was isolated from a C. elegans genomic λ phage library (Stratagene) probing with both 2.0 and 2.2 kb EcoRI fragments of C02H7, which are not contained in M02A10 or R04F4.

PH13 and PH15 were subclones of BglII fragments from C02H7 using the λ DASH II vector (Stratagene). For construction of pTJ500, an 8 kb HindIII to Nod (vector site) fragment from PH15 was cloned into pBluescript SK+ (Stratagene). For construction of pTJ501, a 5.2 kb HindIII to SphI fragment from pTJ500 was cloned into pBluescript SK+. The SphI site was converted to a BamHI site by T4 DNA polymerase treatment and BamHI linker ligation.

aex-3 plasmids carrying frameshift mutations were constructed as follows. pTJ500 was digested with Thh111I, treated with T4 DNA polymerase, and self-ligated, resulting in a 1 base insertion at codon 418 of AEX-3 (pTJ 671). Similarly, pTJ500 was digested with SpeI, treated with T4 DNA polymerase, and self-ligated, resulting in a 4 base deletion at codon 687 of AEX-3 (pTJ 666). DNA sequencing confirmed the frameshifts in both plasmids.

Total RNA from mixed-stage worms was a gift from D. Bimby. Poly(A)-RNA was isolated by oligo-d(T) cellulose selection. RNA was fractionated by agarose gel electrophoresis and transferred to a Nitran membrane (Schleicher and Schuell) by capillary transfer. Hybridization and washing followed a manual supplied by Schleicher and Schuell. The C. elegans cDNA library was a gift from R. Barstead (Barstead et al., 1991).

A cDNA clone was isolated by in situ hybridization using the pTJ 500 insert as a probe. A Bluescript plasmid containing the cDNA insert was generated by the M13 phage excision system (Stratagene). RT-PCR was performed using a 5’ RACE system (Life Technology). Total C. elegans RNA (1 μg) was used to generate first strand cDNA with aex-3-specific primers, OLG296, OLG297, and OLG298. For internal segments of the cDNA, first strand cDNA was amplified by PCR using aex-3-specific primers as described below. For the 5’ end of aex-3, the anchor primer provided with the 5’ RACE system was used. In each case, Southern blot hybridization probing with the pTJ 500 insert confirmed that the RT-PCR products were specific to aex-3. RT-PCR products were cloned into a Bluescript SK+ plasmid. Primer pairs used were as follows: anchor primer-OLG289, OLG290-OLG291, OLG292-OLG293, and OLG294-OLG295. The anchor primer and OLG289-295 (except for OLG294) introduced restriction sites (either Sall, EcoRI, or BamHI), which were used to clone the RT-PCR products. Cloning of PCR products with primers OLG292-OLG293 and OLG294-OLG295 was performed using endogenous EcoRI or BamHI sites on the primers. Primer sequences were:

OLG289—GGAAATTCGTTTTTGGTGAGCACATC
OLG290—CGGATCCAGACGCTCAACTCTAAAG
OLG291—GGAAATTCGTTTTTGGTGAGCACATC
OLG292—CGGATCCAGACGCTCAACTCTAAAG

Figure 7. Expression of an aex-3::gfp Fusion Construct
The top panels are Nomarski images of animals carrying pTJ 644 (an aex-3::gfp construct; see Figure SC). The bottom panels are epifluorescence images of the corresponding regions of the same animals.

(A and B) The head ganglia that surround the nerve ring.
(C and D) A middle segment of body with the ventral nerve cord on the ventral aspect.
(E and F) A posterior tail ganglion. The animal in (C) and (D) is older than those of other panels.
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OLG293—GGAATTCAGCTTCTTCACTCCATACG
OLG294—GATGCTTCCGATACACCTAC
OLG295—CGGATCTTTTGGCTGCTCGGTAATGG
OLG296—TGGCGTAAAAACGGAGGAGG
OLG297—CGCTTCACTAACAAGGTG
OLG298—TTTCCGAGGATTTATCAC

DNA sequencing was performed using a dye-terminator sequencing system (Perkin Elmer). Sequences of the many sequencing primers will be provided upon request. Products of sequencing reactions were analyzed by the Pharmacology Core facility at the University of Washington. Both strands of the cDNA were sequenced at least once. At least three independent clones of RT–PCR products were sequenced on both strands. These sequences were also confirmed by genomic sequences produced by the C. elegans genome project.

The aex-3:gfp fusion plasmid pTJ 644 was constructed as follows. The HindIII–KpnI fragment (1.4 kb long) of pTJ500 was cloned into the HindIII and BamHI sites of pBluescript SK. The HindIII–KpnI fragment (1.4 kb long) of pTJ500 was cloned into the HindIII and BamHI sites of pBluescript SK in the HindIII and BamHI sites of pBluescript SK + (the KpnI site of pTJ 500 was converted to a BamHI site by treatment with T4 DNA polymerase and ligation of a BamHI linker). This HindIII–BamHI fragment was further subcloned into pDP95.69 (a GFP expression vector; a gift from A. Fire). Sequencing confirmed that codon 49 of aex-3 was fused in frame to gfp.

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References


**GenBank Accession Number**

The GenBank accession number for aex-3 sequence is U93842.

**Note Added in Proof**

A paper describing the purification of a protein that is nearly identical to DENN and 35% identical to aex-3 has recently been published (Wada, M., Nakanishi, H., Satoh, A., Hirano, H., Obashi, H., Matsuura, Y., and Takai, Y. (1997). Isolation and characterization of a GDP/GTP exchange protein specific for the Rab3 subfamily small G proteins. J. Biol. Chem. 272, 3875-3878). This protein is shown to have guanine-nucleotide exchange activity specifically for Rab3A, Rab3C, and Rab3D, indicating that AEX-3 is likely to have a similar function for RAB-3.