UNC-73/Trio RhoGEF-2 Activity Modulates *Caenorhabditis elegans* Motility Through Changes in Neurotransmitter Signaling Upstream of the GSA-1/Gα_s Pathway

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ABSTRACT Rho-family GTPases play regulatory roles in many fundamental cellular processes. *Caenorhabditis elegans* UNC-73 RhoGEF isoforms function in axon guidance, cell migration, muscle arm extension, phagocytosis, and neurotransmission by activating either Rac or Rho GTPase subfamilies. Multiple differentially expressed UNC-73 isoforms contain a Rac-specific RhoGEF-1 domain, a Rho-specific RhoGEF-2 domain, or both domains. The UNC-73E RhoGEF-2 isoform is activated by the G-protein subunit Gα_q and is required for normal rates of locomotion; however, mechanisms of UNC-73 and Rho pathway regulation of locomotion are not clear. To better define UNC-73 function in the regulation of motility we used cell-specific and inducible promoters to examine the temporal and spatial requirements of UNC-73 RhoGEF-2 isoform function in mutant rescue experiments. We found that UNC-73E acts within peptidergic neurons of mature animals to regulate locomotion rate. Although *unc-73* RhoGEF-2 mutants have grossly normal synaptic morphology and weak resistance to the acetylcholinesterase inhibitor aldicarb, they are significantly hypersensitive to the acetylcholine receptor agonist levamisole, indicating alterations in acetylcholine neurotransmitter signaling. Consistent with peptidergic neuron function, *unc-73* RhoGEF-2 mutants exhibit a decreased level of neuropeptide release from motor neuron dense core vesicles (DCVs). The *unc-73* locomotory phenotype is similar to those of *rab-2* and *unc-31*, genes with distinct roles in the DCV-mediated secretory pathway. We observed that constitutively active Gα_s pathway mutations, which compensate for DCV-mediated signaling defects, rescue *unc-73* RhoGEF-2 and *rab-2* lethargic movement phenotypes. Together, these data suggest UNC-73 RhoGEF-2 isoforms are required for proper neurotransmitter signaling and may function in the DCV-mediated neuromodulatory regulation of locomotion rate.

THE Rho GTPase signaling pathway is involved in multiple cellular processes such as gene transcription, cytokinesis, cell-cycle progression, and regulation of the actin cytoskeleton and more recently was found to play a role in neurotransmission (Jaffe and Hall 2005; Steven *et al.* 2005; McMullan *et al.* 2006; Williams *et al.* 2007). In *Caenorhabditis elegans* Rho upregulates neurotransmission in at least two ways. GTP-bound Rho, activated by the Rho **g**uanine nucleotide **e**xchange **f**actor (RhoGEF) RHGF-1, binds to and inhibits diacylglycerol kinase (DGK), which results in increased levels of its substrate diacylglycerol (DAG) at the synapse (Hiley *et al.* 2006; McMullan *et al.* 2006). Increased DAG facilitates the release of neurotransmitter likely due to an accumulation of UNC-13, a component of the synaptic vesicle release machinery, via UNC-13 binding to DAG in the synaptic membrane (Lackner *et al.* 1999). Increased acetylcholine release through the Rho pathway ultimately leads to an increased rate of locomotion (McMullan *et al.* 2006).

Rho also affects neurotransmission in a DGK– and UNC-13–independent manner since inactivation of Rho in a *dgk-1* mutant background still decreases locomotion rate and an UNC-13 DAG-binding mutant only partially blocks constitutive RHO-1 activity (McMullan *et al.* 2006). UNC-73, the *C. elegans* homolog of mammalian Trio and Kalirin, is a candidate RhoGEF acting in the DGK-independent pathway (Debant *et al.* 1996; Alam *et al.* 1997; Steven *et al.* 2005;

doi: 10.1534/genetics.111.131227
Manuscript received December 17, 2011; accepted for publication June 20, 2011
Supporting information is available online at http://www.genetics.org/content/suppl/2011/07/13/genetics.111.131227.DC2.
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Hiley *et al.* 2006). The *unc-73* gene encodes multiple isoforms, all of which contain at least one RhoGEF domain, indicating they are potential activators of Rho family GTPases. The smaller UNC-73 isoforms contain either a RhoGEF-1 domain, which specifically activates Rac family GTPases in biochemical assays (Steven *et al.* 1998; Wu *et al.* 2002; Kubiseski *et al.* 2003), or a RhoGEF-2 domain, which is specific to Rho (Spencer *et al.* 2001). The UNC-73B RhoGEF-1 isoform and the Rac pathways have well-characterized roles in axon guidance, cell migration, muscle arm extension, and the phagocytosis of apoptotic cells, while the functions of the UNC-73 RhoGEF-2 isoforms are the focus of this study (Hedgecock *et al.* 1987; Steven *et al.* 1998; Honigberg and Kenyon 2000; Kishore and Sundaram 2002; Lundquist 2003; Debakker *et al.* 2004; Morita *et al.* 2005; Levy-Strumpf and Culotti 2007; Watari-Goshima *et al.* 2007; Axang *et al.* 2008; Alexander *et al.* 2009). Isoform-specific rescue experiments previously revealed the RhoGEF-2 domain-containing UNC-73C1, C2/F, and E isoforms act redundantly to regulate the speed of locomotion (Steven *et al.* 2005). Transgenic animals lacking these specific isoforms have a lethargic movement phenotype that is rescued by the expression of any individual isoform from the group. Point mutations that reduce UNC-73 RhoGEF-2 activity also result in a lethargic phenotype (Steven *et al.* 2005; Williams *et al.* 2007), but deletion of the entire RhoGEF-2 domain causes early larval arrest as a result of pharynx pumping defects (Steven *et al.* 2005).

Analysis of *C. elegans* heterotrimeric G-protein signaling revealed a network of $G\alpha_q$, $G\alpha_o$, and $G\alpha_s$ pathways regulating acetylcholine release from motor neurons (Perez-Mansilla and Nurrish 2009). $G\alpha_q$ (*C. elegans* EGL-30) and $G\alpha_s$ (GSA-1) positively influence neurotransmitter release while $G\alpha_o$ (GOA-1) activity reduces neurotransmitter release through inhibition of the $G\alpha_q$/EGL-30 pathway (Hajdu-Cronin *et al.* 1999; Lackner *et al.* 1999; Miller *et al.* 1999; Reynolds *et al.* 2005; Schade *et al.* 2005; Charlie *et al.* 2006). Our previous genetic analysis placed *unc-73* RhoGEF-2 activity downstream of $G\alpha_o$/GOA-1 and Williams *et al.* (2007) more specifically identified the UNC-73E RhoGEF-2 isoform as a $G\alpha_q$/EGL-30 effector acting in parallel to phospholipase C_β (EGL-8) (Steven *et al.* 2005). In mammals, $G\alpha_q$ similarly interacts with the Rho-specific activators p63RhoGEF and Trio, an UNC-73 homolog (Lutz *et al.* 2005; Rojas *et al.* 2007). *C. elegans egl-30* and *unc-73* RhoGEF-2 loss-of-function mutations have lethargic or slow movement phenotypes that are opposite to the hyperactive movement phenotype of transgenic animals carrying a Rho gain-of-function mutation (Brundage *et al.* 1996; Steven *et al.* 2005; McMullan *et al.* 2006; Williams *et al.* 2007). However, the precise role of UNC-73 RhoGEF-2–stimulated Rho activity in the regulation of locomotion is not known.

Neurotransmission involves two separate mechanisms of neurotransmitter release (Sudhof 2008). Classical small molecule neurotransmitters such as acetylcholine and glutamate are stored in small, clear vesicles that are filled and released at presynaptic sites. In response to presynaptic depolarization the vesicles fuse to the plasma membrane and neurotransmitters are released into the synapse where they have immediate effects to initiate depolarization in the postsynaptic neuron. Neuromodulatory neurotransmitters such as neuropeptides, on the other hand, are held in large, dense core vesicles (DCVs) produced by the Golgi apparatus in the cell body. They are transported down the axon for exocytosis at the synapse, as well as other sites, where they tend to have slower and longer-lasting effects on the nervous system compared to classical neurotransmitters (Katz and Frost 1996; Husson *et al.* 2007).

The results of this study suggest UNC-73 RhoGEF-2 isoforms affect *C. elegans* motility through the modulation of neurotransmission signaling mechanisms. *unc-73* RhoGEF-2 mutants are weakly resistant to the acetylcholinesterase inhibitor aldicarb and are hypersensitive to the acetylcholine agonist levamisole, indicating alterations in cholinergic signaling. DCV-mediated neuropeptide signaling is also altered in *unc-73* RhoGEF-2 mutants and the *unc-73* RhoGEF-2 lethargic movement phenotype is very similar to the locomotory phenotypes of *rab-2* and *unc-31*, two genes with established roles in DCV-mediated signaling (Avery *et al.* 1993; Speese *et al.* 2007; Chun *et al.* 2008; Edwards *et al.* 2009; Sumakovic *et al.* 2009). Consistent with these results we observe that activating mutations in the $G\alpha_s$/GSA-1 pathway, which increase DCV exocytosis and compensate for neuropeptide release defects in *C. elegans* (Charlie *et al.* 2006; Zhou *et al.* 2007), rescue the *unc-73* and *rab-2* lethargic movement phenotypes. Our data suggest that UNC-73 RhoGEF-2 isoforms and the Rho GTPase pathway alter DCV-mediated signaling involving neuropeptides or other neuromodulatory molecules that stimulate locomotion in *C. elegans*.

Materials and Methods

C. elegans strains

Strains were maintained at 21° on plates containing standard nematode growth media unless otherwise noted. The following strains were used in this study: N2 Bristol (wild type), NL3231 *acy-1(pk484)* III; *dpy-20(e1362)* IV; *pkIs296[hsp*::*gsa-1(QL)*; *dpy-20(+)]* X (Korswagen *et al.* 1997); KG518 *acy-1(ce2)*, VC671 *egl-3(ok979)*, RM2221 *egl-8 (md1971)*, *egl-30(tg26)* (Doi and Iwasaki 2002); KG421 *gsa-1(ce81)*, *juIs1[unc-25p*::*snb-1*::*gfp]* (Hallam and Jin 1998); KG532 *kin-2(ce179)*, *nuIs183[unc-129p*::*nlp-21*::*YFP*; *myo-2p*::*NLS*::*gfp]* IV (Sieburth *et al.* 2007); QT47 *nzIs1*[*HSp*::*rho-1(gf)*; *ttx-3p*::*gfp*] I (McMullan *et al.* 2006); MT1093 *rab-2(n501)* (*rab-2* is also known as *unc-108*), XA7314 *unc-73(ev802)* I; *qaIs7312[unc-73D1*; *F25B3.3p*::*gfp]* V, XA7330 *qaEx7327 [unc-73D1*; *unc-73E*; *F25B3.3p*::*gfp]*; *unc-73(ev802)*, CB928 *unc-31(e928)*, XA7300 *unc-73(ev802)/unc-11(e47) dpy-5 (e61)*, and KG1278 *unc-73(ce362)*. Double-mutant strains were constructed using standard genetic methods without additional marker mutations. Mutations in the double mutants

were confirmed by PCR, sequencing, or outcrossing to *him-5* males and examination of the F_2 progeny phenotypes.

Transgenic lines

Standard microinjection techniques were used to generate stable transgenic *C. elegans* lines carrying extrachromosomal DNA arrays. For the *unc-73(ev802)* rescue experiments, between 50 and 100 ng/μl of the *gene-Xp*::*unc-73E*::*gfp* constructs were mixed with 50–100 ng/μl of pXS2 encoding *unc-73D1* and injected into *unc-73(ev802)/unc-11 dpy-5* without a cotransformation marker. pXS2 was added to rescue *unc-73(ev802)* lethality. Progeny were screened for stable expression of the extrachromosomal array and homozygous *unc-73(ev802)* lines containing the array were established. For *unc-73(ce362)* rescue experiments, transgenic lines were first established in a wild-type background, by injection of 100 ng/μl *gene-Xp*::*unc-73E*::*gfp* without a cotransformation marker, and then the lines were crossed into *unc-73(ce362)*. Exceptions were the *dat-1p* and *eat-4p* constructs, which were injected into wild type at 50 ng/μl along with 50 ng/μl *unc-122p*::*gfp* (coelomocyte-specific promoter) as a cotransformation marker. PCR-based genotyping was used to confirm *unc-73(ev802)* and *unc-73 (ce362)* rescued lines were homozygous.

Gain-of-function *acy-1* cDNA constructs [KG#81 *myo-3p*::*acy-1(gf)* and KG#83 *rab-3p*::*acy-1(gf)*] (Reynolds *et al.* 2005) at 10 ng/μl with 50 ng/μl *unc-122p*::*gfp* and 90 ng/μl herring sperm DNA were injected into N2 to establish stable transgenic lines that were crossed into *unc-73(ce362)*. The synapse marker KP#282 *acr-2p*::*snb-1*::*cfp* (Nurrish *et al.* 1999) at 50 ng/μl mixed with 50 ng/μl pRF4 *rol-6 (su1006dm)* and 50 ng/μl herring sperm DNA were injected into N2 and *unc-73(ce362)* animals to establish stable lines. The plasmid KP#315 *acr-2p*::*egl-30(Q209L)* (Lackner *et al.* 1999) at 10 ng/μl mixed with 50 ng/μl *F25B3.3p*::*gfp* (a cotransformation marker expressed in neurons) and 70 ng/μl herring sperm DNA were injected into N2 and *unc-73(ce362)* to establish stable transgenic lines. At least three transgenic lines were isolated for each injected plasmid mix and it was confirmed that each set of lines had the same phenotype and/or GFP expression pattern.

Molecular biology

The *gene-Xp*::*unc-73E*::*gfp* cell-specific promoter constructs were made by directionally cloning promoter DNA into the unique *Not*I (5′) and *Eco*RV or *Eco*RI (3′) sites of the *unc-73E*::*gfp* plasmid pXS6. In this construct the *unc-73E* transcript is encoded by cDNA for the first two exons with the remainder encoded by a genomic DNA fragment extending ~150 bp beyond the 3′ end of the coding region of the transcript. Promoters generated by PCR using wild-type genomic DNA or the plasmids pPD49.83 (heat-shock promoter; from Andy Fire, Stanford University, Palo Alto, CA), pPD136.64 (*myo-3* promoter; from Andy Fire), or pRM621 (modified *unc-17* promoter as described in Charlie *et al.* 2006; from Jim Rand, Oklahoma Medical Research Foundation, Oklahoma City, OK) as the template were cloned into pJET (Fermentas) or pGEM (Promega, Madison, WI) PCR cloning vectors and confirmed by sequencing. We used the same promoter regions previously defined in mutant rescue experiments (Lee *et al.* 1999; Nass *et al.* 2002; Carvelli *et al.* 2004; Carnell *et al.* 2005; Liu *et al.* 2007). Two promoters were not generated by PCR, but directly cloned from plasmids. pBY103 (from Dave Pilgrim, University of Alberta, AB, Canada) contains the 1.2-kb *Hin*dIII/*Eco*RI *unc-119* promoter fragment and pJL35 (from Bruce Bamber, University of Toledo, Toledo, OH) contains the 1.2-kb *unc-47* promoter fragment.

Phenotype analysis

The aldicarb sensitivity protocol was modified from Mahoney *et al.* (2006). Larval stage four (L4) worms were picked the day before the assay was performed and left to grow overnight on plates with nematode growth media (NGM) and bacteria. The next day the young adults were transferred to NGM plates containing 1.0 mM aldicarb (Chem Services). Plates were stored at 4° for 1 to 5 days and seeded with bacteria the day of use. Approximately 30 animals per strain were examined blind for paralysis every 10 min for 2 hr, using a Leica MZ6 stereomicroscope. Animals were defined as paralyzed if after a maximum of three worm pick taps on the head and three taps on the tail there was no body movement; however, animals exhibiting only small foraging movements of the head or small movements of the tail were still considered paralyzed. The assay was repeated three times for each strain and an average for each time point was calculated.

Levamisole sensitivity assays were performed as described above for aldicarb sensitivity. Staged young adult worms were examined on seeded NGM plates containing 0.5 mM levamisole (Sigma, St. Louis) over a period of 1 hr. Plates were prepared as described above for aldicarb and at least three trials were performed for each strain.

Rates of locomotion were determined at room temperature (~21°) as described previously (Steven *et al.* 2005). Healthy and fed young adult animals were transferred to NGM assay plates without bacteria and left for at least 20 sec before counting. Locomotion rate is defined as the number of body bends exhibited in 20 sec of uninterrupted forward movement. If an animal stopped moving or reversed direction, the count was abandoned. One body bend was defined as a complete cycle of terminal bulb motion starting from the top position of the sinusoidal wave track through to the bottom and back to the top. Between 25 and 30 animals were examined for each strain.

NGM plates containing the phorbol ester phorbol 12-myristate 13 acetate (PMA) (Enzo Life Sciences) were made as described by Reynolds *et al.* (2005). Approximately 15–20 adult worms for each strain were placed on separate PMA or control (containing the ethanol carrier) plates at room temperature and observed 2.5–3 hr later. PMA treatment of the lethargic *unc-73* and *rab-2* mutants increased the

locomotion rates of these mutants, but they tended to coil and reverse frequently, which made it impossible to measure their locomotion rates in our body bend assays. Video images were captured with a Dino-Lite AM413T digital microscope in a "Worm Tracker" rig and processed using Worm Tracker 2.0 software (Schafer Lab).

Coelomocyte endocytosis in *C. elegans* strains was examined by monitoring Texas Red-conjugated bovine serum albumin (TR-BSA) (Invitrogen) uptake by coelomocytes (Zhang *et al.* 2001). Synchronized young adults were injected with a short pulse of 1 mg/ml TR-BSA into the pseudocoelom in the region of the pharynx between the metacorpus and the terminal bulb. Animals were left to recover on seeded NGM plates for 1 hr and then collected in a drop of ice-cold 1% paraformaldehyde (Electron Microscopy Sciences) in M9 on a slide with a 2% agarose pad. They were maintained in the drop for at least 20 min and up to 2 hr, on ice, as all the injected worms were collected, and then covered with a coverslip and observed under the microscope.

Microscopy and imaging analysis

Worms were immobilized with 30 mg/ml 2,3-butanedione monoxime (BDM) (Sigma) or 10 mM levamisole (Sigma) in M9. Images were captured with a Hamamatsu ORCA-ER camera mounted on a Leica DMRA2 microscope and processed using Openlab software (Improvision, Lexington, MA) or captured with a QICAM camera (QImaging) mounted on a Zeiss Axiophot microscope and processed with Q Capture Pro (QImaging). Confocal microscopy images were obtained with an Olympus Fluoview 300/IX70 confocal microscope. For NLP-21::YFP and TR-BSA analysis stacks of 0.4-μm-thick optical images were captured with Olympus Fluoview 5.0 software and fluorescence intensity was quantified using Volocity software (Improvision). Images were tightly cropped to contain the cells or axons of interest and fluorescent objects within the images were identified automatically by intensity. The threshold value for this process was kept constant for each experiment and was appropriately chosen to eliminate "background" fluorescence as observed in wild-type animals. Object intensities were quantified and summed for each image. Small objects containing <30 voxels (coelomocytes), 6 voxels (cell bodies), or 4 voxels (dorsal axons) were eliminated since these likely corresponded to nonspecific background specks. The arbitrary fluorescent unit of each measurement was standardized to the average fluorescent unit of wild type obtained for that day.

Synchronized young adults with dorsal cord axon bundles oriented toward the objective were imaged using a UPlanApo 40× objective. The fluorescent intensity was quantified as the arbitrary fluorescent unit per unit length. The DA6 and DB6 cell bodies in the region posterior to the vulva were imaged with a UPlanApo 100× objective in synchronized young adults with the ventral cord oriented toward the objective. Fluorescence intensity was quantified as the arbitrary fluorescent unit per two cell bodies. Single posterior coelomocytes oriented toward the objective were imaged in L4 larvae using the UPlanApo 100× objective. Fluorescence intensity was quantified as the arbitrary fluorescent unit per coelomocyte.

Results

UNC-73 RhoGEF-2 isoforms modulate acetylcholine signaling

To better understand how UNC-73 RhoGEF-2 isoforms regulate locomotion we first checked for defects in classical synaptic vesicle neurotransmission. Measuring resistance to the acetylcholinesterase inhibitor aldicarb is an indirect method of examining alterations in *C. elegans* cholinergic signaling. Aldicarb exposure increases the amount of synaptic acetylcholine, which paralyzes worms (Nguyen *et al.* 1995). Control *egl-8* mutants, with defective phospholipase C, release less acetylcholine into the synapse and were therefore moderately resistant to aldicarb in comparison to wild-type animals (Figure 1A) (Lackner *et al.* 1999). We tested two *unc-73* mutants for aldicarb resistance. *unc-73(ce362)* animals have a point mutation in the RhoGEF-2 domain that severely reduces RhoGEF activity (Williams *et al.* 2007). Transgenic *Is[D1]*; *unc-73 (ev802)* animals have a deletion eliminating the RhoGEF-2 domain but they express the UNC-73D1 RhoGEF-2 isoform in the pharynx under the control of the endogenous *unc-73D1* promoter, which rescues the *unc-73(ev802)* larval arrest phenotype (Steven *et al.* 2005). The *unc-73(ce362)* and *Is[D1]*; *unc-73(ev802)* strains both exhibited a lethargic movement phenotype (Figure 2) (Steven *et al.* 2005; Williams *et al.* 2007). Wild-type (N2) and transgenic *Ex[D1]*; *E]*; *unc-73 (ev802)* and *Ex[E]*; *unc-73(ce362)* rescued animals, which have wild-type rates of movement, served as controls (Figure 2) (Steven *et al.* 2005; Williams *et al.* 2007). Both *unc-73* mutants exhibited weak aldicarb resistance in comparison to the control animals (Figure 1, A and B), indicating *unc-73* RhoGEF-2 mutants have altered acetylcholine signaling consistent with a minor or modulatory role for RhoGEF-2 activity in cholinergic neurotransmission.

Aldicarb resistance may result from lower levels of acetylcholine release at synapses or a diminished postsynaptic response to acetylcholine. The postsynaptic response to acetylcholine was examined in *unc-73* mutants using the acetylcholine receptor agonist levamisole (Lewis *et al.* 1980). *unc-73(ce362)* and *Is[D1]*; *unc-73(ev802)* animals did not have a reduced levamisole response and were actually hypersensitive to the drug (Figure 1D) (Williams *et al.* 2007). Levamisole hypersensitivity was reduced in control *unc-73* rescued strains (Figure 1D). Therefore, *unc-73* aldicarb resistance and lethargic movement phenotypes are not due to a reduced response to acetylcholine by body wall muscles and it is possible a more significant *unc-73* aldicarb resistance phenotype is masked by an increased sensitivity to acetylcholine. Interestingly, other genes with roles in neurotransmission including *snt-1*, *unc-18*, *unc-41*,

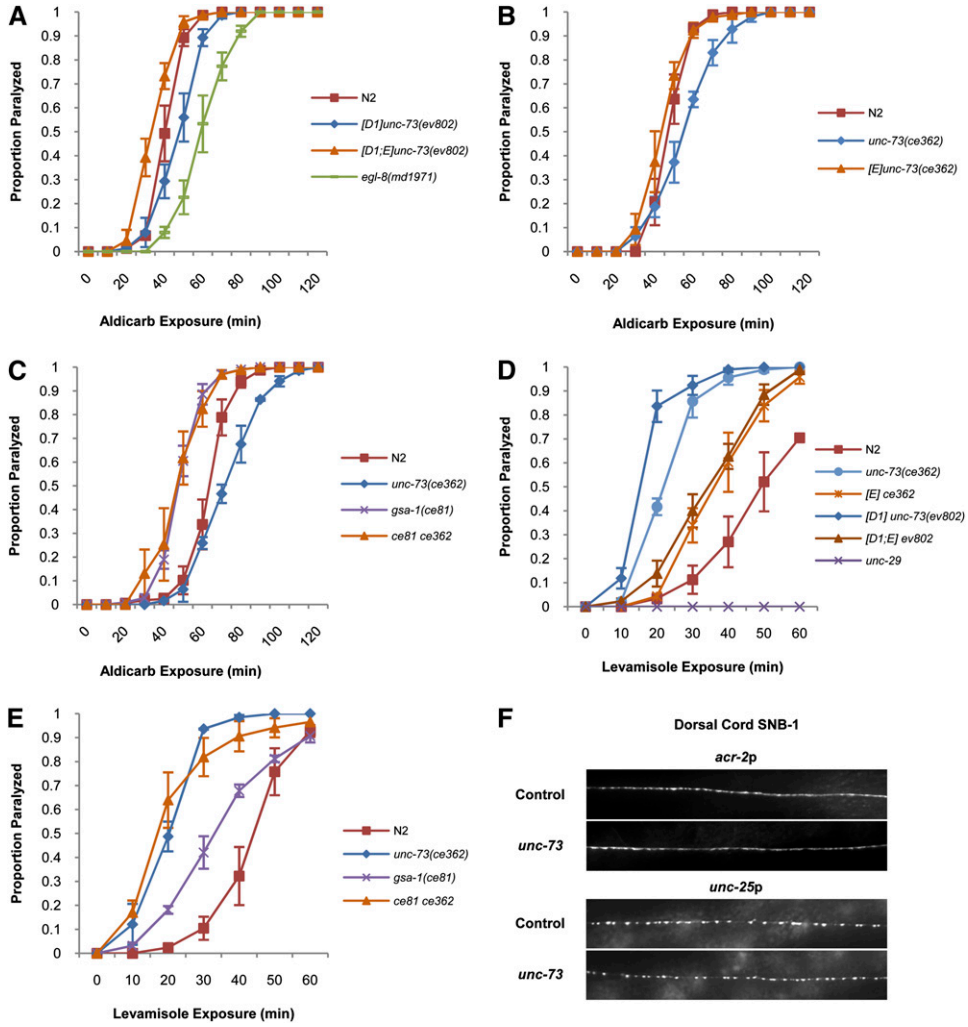

Figure 1 *unc-73* RhoGEF-2 mutants have altered neurotransmitter signaling. (A–C) Animals were examined for paralysis on NGM plates containing 1 mM aldicarb, an acetylcholinesterase inhibitor. *unc-73* RhoGEF-2 mutants are weakly resistant to aldicarb compared to wild-type N2 animals and *Ex[D1; E]*; *unc-73 (ev802)* and *Ex[E]*; *unc-73(ce362)* rescued strains. Control *egl-8* mutants are moderately resistant to aldicarb. *gsa-1 (ce81)* gain-of-function mutants and *gsa-1(ce81) unc-73(ce362)* double mutants are hypersensitive to aldicarb. (D and E) Animals were examined for paralysis on NGM plates containing 0.5 mM levamisole, an acetylcholine receptor agonist. *unc-73* RhoGEF-2 mutants are hypersensitive to levamisole. Control *unc-29* levamisole-sensitive acetylcholine receptor subunit mutants are completely resistant. Error bars indicate SEM. Square brackets denote transgenes present in a particular strain. For example, [D1; E] indicates the *unc-73D1* and *unc-73E* isoform encoding transgenes. (F) Gross synapse morphology is unchanged in *unc-73* RhoGEF-2 mutants. SNB-1::CFP (synaptobrevin) localization in dorsal cord cholinergic motor neuron axons [*acr-2* promoter (Nurrish *et al.* 1999)] is similar in *unc-73(ce362)* mutants and control N2 animals (top). SNB-1::GFP localization in dorsal cord GABAergic motor neuron axons [*unc-25* promoter (Hallam and Jin 1998)] is similar in *Is[D1]*; *unc-73 (ev802)* mutants and control *unc-73 (ev802)*/*unc-11 dpy-5* animals (bottom).

and *unc-75* also display levamisole hypersensitivity phenotypes for unknown reasons, but possibly due to receptor upregulation to compensate for neurotransmission defects (Miller *et al.* 1996).

Defects in neurotransmission and locomotory behavior may result from structural defects within the nervous system. Synapse structure was examined on a gross level in *Is[D1]*; *unc-73(ev802)* and *unc-73(ce362)* mutants using fluorophore-tagged SNB-1, which localizes to presynaptic vesicle clusters in neurons (Nonet 1999). Tagged SNB-1 was examined in *unc-73* mutant GABAergic and cholinergic motor neurons using *unc-25* and *acr-2* promoters, respectively (Figure 1F). The size and spacing of the vesicle clusters in dorsal cord axons was similar in *unc-73* mutants and control animals, suggesting *unc-73* RhoGEF-2 isoforms are not required for normal presynaptic structure.

unc-73 RhoGEF-2 isoforms function in adult neurons to regulate locomotion

UNC-73 RhoGEF-2 isoforms that rescue the *Is[D1]*; *unc-73 (ev802)* lethargic phenotype have differential expression patterns, but they are all expressed to some extent in the nervous system (Steven *et al.* 2005). We used UNC-73E as a representative RhoGEF-2 isoform in tissue-specific rescue experiments to better define where UNC-73 RhoGEF-2 activity is required for wild-type rates of locomotion. UNC-73E expression in the nervous system (*unc-119* promoter), but not in the body wall muscles (*myo-3* promoter) rescued the *Is[D1]*; *unc-73(ev802)* lethargic movement phenotype (Figure 2). *Ex[unc-119p*::*unc-73E*::*gfp*; *D1]*; *unc-73(ev802)* animals moved at a rate comparable to that of wild-type N2, while the movement of *Ex[myo-3p*::*unc-73E*::*gfp*; *D1]*; *unc-73(ev802)* animals was not significantly different from that of lethargic *Is[D1]*; *unc-73(ev802)* animals. The *unc-73D1* transgene was included in these transgenic strains to rescue the lethality of the *unc-73(ev802)* allele. UNC-73E::GFP fusion protein expression in the expected tissues was confirmed in all of our transgenic strains by fluorescence microscopy (Supporting Information, Figure S2 and Figure S3).

To assess the temporal requirements of UNC-73E expression for normal locomotion we used a heat-shock promoter (*hsp-16.41*) to control UNC-73E expression. If *Is[D1]*; *unc-*

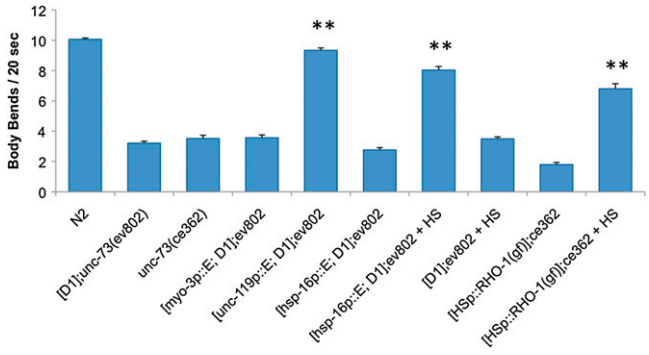

Figure 2 UNC-73E functions in the adult nervous system to regulate locomotion. Expression of UNC-73E in the nervous system (*unc-119* promoter), but not body wall muscles (*myo-3* promoter) rescues *Is[D1]*; *unc-73 (ev802)* lethargy. Heat-shock (HS)-induced UNC-73E or RHO-1(gf) expression (using the *hsp-16* promoter) in adult animals also rescues *unc-73* RhoGEF-2 mutant locomotion defects. Error bars indicate SEM. $^{**}P < 0.001$ in comparison to *Is[D1]*; *unc-73(ev802)* or *unc-73(ce362)* using Student's *t*-test.

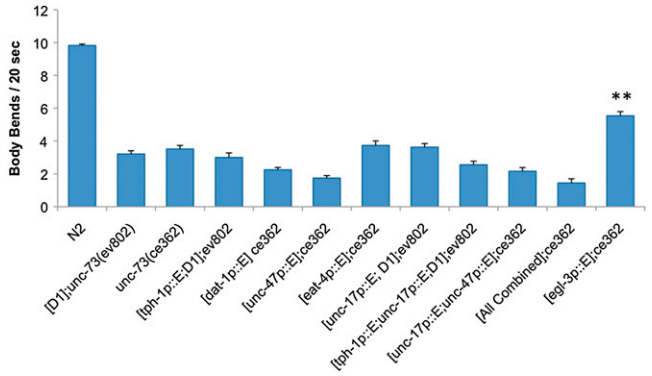

Figure 3 UNC-73E expression in peptidergic neurons, but not other neuronal subtypes, rescues *unc-73* RhoGEF-2 mutant lethargy. UNC-73E expression in ventral cord cholinergic (*unc-17* promoter), dopaminergic (*dat-1* promoter), GABAergic (*unc-47* promoter), glutamatergic (*eat-4* promoter), or serotonergic neurons (*tph-1* promoter) alone or in the indicated combinations, including all constructs combined ("all combined"), failed to rescue *Is[D1]*; *unc-73(ev802)* or *unc-73(ce362)* locomotory defects. However, partial rescue was obtained using the *egl-3* promoter, which drives expression in peptidergic neurons. Error bars indicate SEM. $^{**}P < 0.001$ in comparison to *Is[D1]*; *unc-73(ev802)* or *unc-73(ce362)* using Student's *t*-test.

73(ev802) lethargy is a result of defects in neurotransmission and not due to developmental defects, such as axon pathfinding errors, then expressing one of the C1, C2, or E isoforms postdevelopment in adult animals should rescue the lethargy. Indeed, a 40-min heat shock of adult *Ex[hsp-16p*::*unc-73E*::*gfp*; *D1]*; *unc-73(ev802)* animals induced UNC-73E expression and restored movement close to wild-type rates, while animals that did not receive the heat shock remained lethargic (Figure 2 and Figure S2D). This indicated UNC-73E, and by implication also the C1 and C2 isoforms, plays a nondevelopmental role in the regulation of locomotion, consistent with a previous report of UNC-73E function in adult neurons observed with a different allele (Williams *et al.* 2007).

Additional promoters were used to examine the cellular requirements of UNC-73E expression for wild-type locomotion. UNC-73E expression in different classes of neurons including dopaminergic, GABAergic, glutamatergic, serotonergic, and cholinergic neurons did not rescue the *Is[D1]*; *unc-73(ev802)* or *unc-73(ce362)* lethargic phenotypes when the constructs were injected individually or together in combination (Figure 3 and Figure S3). In contrast, UNC-73E expression in peptidergic neurons using the *egl-3* promoter (Kass *et al.* 2001) did rescue *unc-73(ce362)* and *Is[D1]*; *unc-73(ev802)* slow movement phenotypes (Figure 3 and data not shown). *egl-3* encodes a proprotein convertase that is copackaged in dense core vesicles with neuropeptides and is expressed in peptidergic neurons, a large subset of neurons, which includes the cholinergic motor neurons (Kass *et al.* 2001). Although UNC-73E expression driven by the *egl-3* promoter was widespread in the nervous system (Figure S3E), these results suggested that UNC-73 RhoGEF-2 isoforms might play a role in peptidergic neurotransmission to influence locomotion rates.

The UNC-73 RhoGEF-2 domain specifically activates the GTPase Rho *in vitro* (Spencer *et al.* 2001; Williams *et al.* 2007). We used a *rho-1* gain-of-function cDNA driven by a heat-shock promoter to examine whether *rho-1* acts downstream of UNC-73 to regulate the rate of locomotion *in vivo*. Heat-shocked adult *Ex[hsp-16p*::*rho-1(gf)]*; *unc-73(ce362)* animals moved approximately three times faster than animals that were not heat-shocked (Figure 2). This partial rescue might be expected since constitutive activation of Rho GTPases can disrupt many cell behaviors and cycling between the active and inactive forms is likely required for normal function (Luo *et al.* 1994; Morita *et al.* 2005). However, our results are consistent with the *in vitro* results and indicate RHO-1 functions downstream of UNC-73 RhoGEF-2 activity to regulate locomotion rate in adult animals *in vivo*.

unc-73 RhoGEF-2 isoforms modulate neuropeptide levels

Our cell-specific rescue experiments revealed that UNC-73E expression in peptidergic neurons rescued *unc-73* mutant lethargy. We used a neuropeptide release assay, recently developed independently by the Kaplan and Jorgensen laboratories, to more directly assess the role of UNC-73 RhoGEF-2 isoforms in peptidergic neurotransmission (Sieburth *et al.* 2007; Speese *et al.* 2007). Fluorescently labeled neuropeptide, NLP-21::YFP, was expressed in DA and DB motor neurons [using the neural *unc-129* promoter (Colavita *et al.* 1998)], which also express UNC-73 RhoGEF-2 isoforms (Steven *et al.* 2005). Neuropeptide release from these neurons was examined indirectly by measuring YFP fluorescence in scavenger coelomocyte cells, which collect secreted proteins from the pseudocoelom. Coelomocyte YFP fluorescence in *unc-73(ce362)* mutants was ~33% lower ($P = 0.01$) than

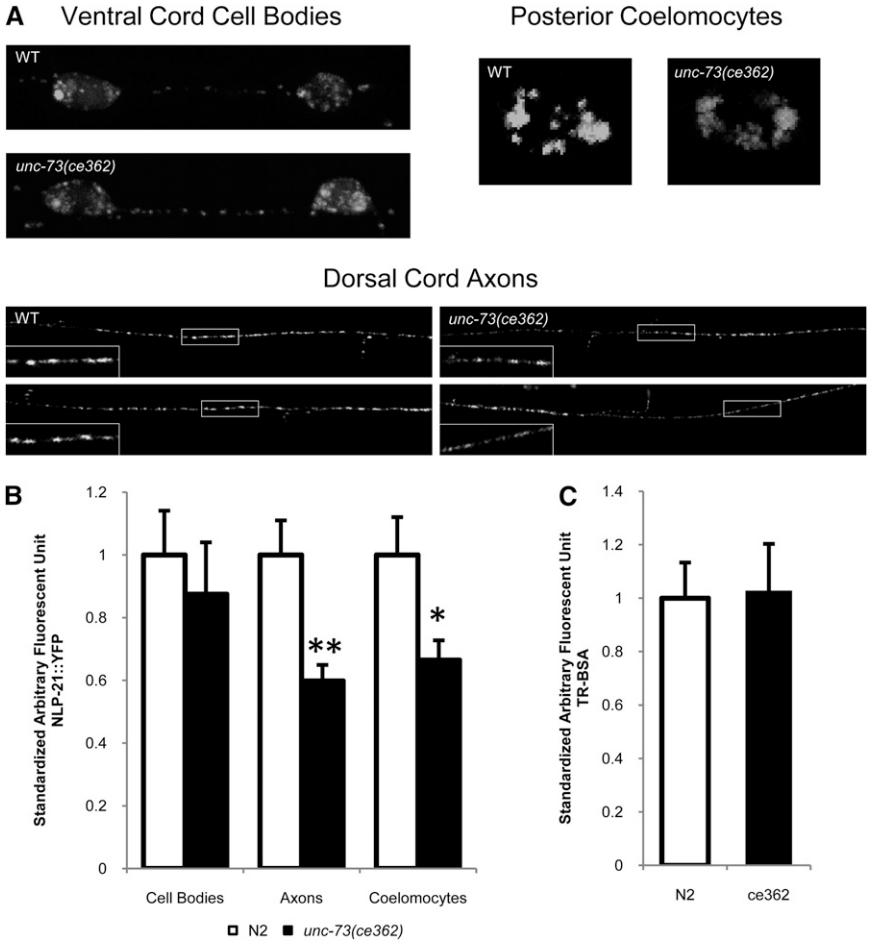

Figure 4 Neuropeptide level is reduced in *unc-73* RhoGEF-2 mutants. NLP-21::YFP expressed in cholinergic motor neurons was used to examine neuropeptide levels and neuropeptide release. (A) Representative images of DA6 and DB6 cholinergic motor neuron cell bodies in the ventral cord, axons in the dorsal cord, and posterior coelomocytes in wild-type and *unc-73(ce362)* animals containing *unc-129p*::*nlp-21*::*yfp*. Insets reveal the punctate localization of NLP-21::YFP in axons. (B) Quantification of the arbitrary fluorescence intensity standardized to wild type. No significant difference between mutant and wild-type DA6 and DB6 cell body fluorescence was observed ($P = 0.58$). YFP fluorescence levels were decreased in *unc-73 (ce362)* dorsal cord axons ($^{**}P < 0.001$) and posterior coelomocyte cells ($^{*}P = 0.01$) in comparison to wild type. (C) Coelomocyte uptake of Texas Red-conjugated bovine serum albumin injected into the pseudocoelom is similar in N2 and *unc-73(ce362)* animals ($P = 0.86$). Error bars show SEM. *P*-values were calculated using Wilcoxon's two-sample test.

the fluorescence from wild-type animals, suggesting less neuropeptide was released from UNC-73 RhoGEF-2 mutant neurons in this assay (Figure 4). Coelomocyte fluorescence after microinjection of fluorescently labeled BSA into the pseudocoelom revealed *unc-73(ce362)* mutants did not have coelomocyte uptake defects that would interfere with the interpretation of the neuropeptide release assay (Figure 4C).

To distinguish whether the reduced neuropeptide release in the *unc-73* mutant was due to defects in the production, transport, or release of the neuropeptide we also examined YFP fluorescence in the axons and cell bodies of NLP-21:: YFP-expressing neurons. YFP fluorescence in wild-type and mutant dorsal cords was punctate as expected from dense core vesicle cotransport of proprotein convertase cleaved neuropeptide and YFP down the axons (Figure 4A) (Kass *et al.* 2001; Sieburth *et al.* 2007). The *unc-73(ce362)* dorsal cord axon fluorescence was 40% lower compared to wild type ($P = 0.001$), but the cell bodies of mutant and wild-type animals had similar fluorescence levels ($P = 0.58$) (Figure 4). We conclude that the UNC-73 RhoGEF-2 isoforms are required for the production or maintenance of wild-type neuropeptide levels in axons, which ultimately affects the amount of neuropeptide released from neurons.

The $G\alpha_s$ pathway functions downstream of UNC-73 RhoGEF-2 activity

To better define where UNC-73 and Rho GTPase signaling may fit in the pathways regulating locomotion we performed an epistasis analysis with *unc-73* lethargic mutants and genes that are known to regulate locomotion in *C. elegans* and display a hyperactive movement phenotype. Double mutants were constructed with either *unc-73(ce362)* or *Is[D1]*; *unc-73(ev802)*, which have similar lethargic movement phenotypes. *unc-73* double mutants made with hyperactive *dgk-1* or *goa-1* loss-of-function mutants were lethargic, suggesting UNC-73 RhoGEF-2 isoforms function downstream of or in parallel to *dgk-1* and *goa-1* (Steven *et al.* 2005). *Is[D1]*; *unc-73(ev802)* lethargy also suppressed the hyperactivity of the *egl-30(tg26)* gain-of-function allele and the hyperactivity of animals expressing a constitutively active *egl-30* cDNA in cholinergic motor neurons, which is consistent with a study identifying UNC-73E as a downstream effector of $G\alpha_q$/EGL-30 (Williams *et al.* 2007) (data not shown). In contrast, mutations that constitutively activate the $G\alpha_s$/GSA-1 pathway, including *gsa-1(ce81)*, *acy-1 (ce2)* (**a**denylyl **cy**clase-1), and *kin-2(ce179)* (protein **kin**ase-2; PKA regulatory subunit) completely suppressed the lethargy of *Is[D1]*; *unc-73(ev802)* and *unc-73(ce362)*

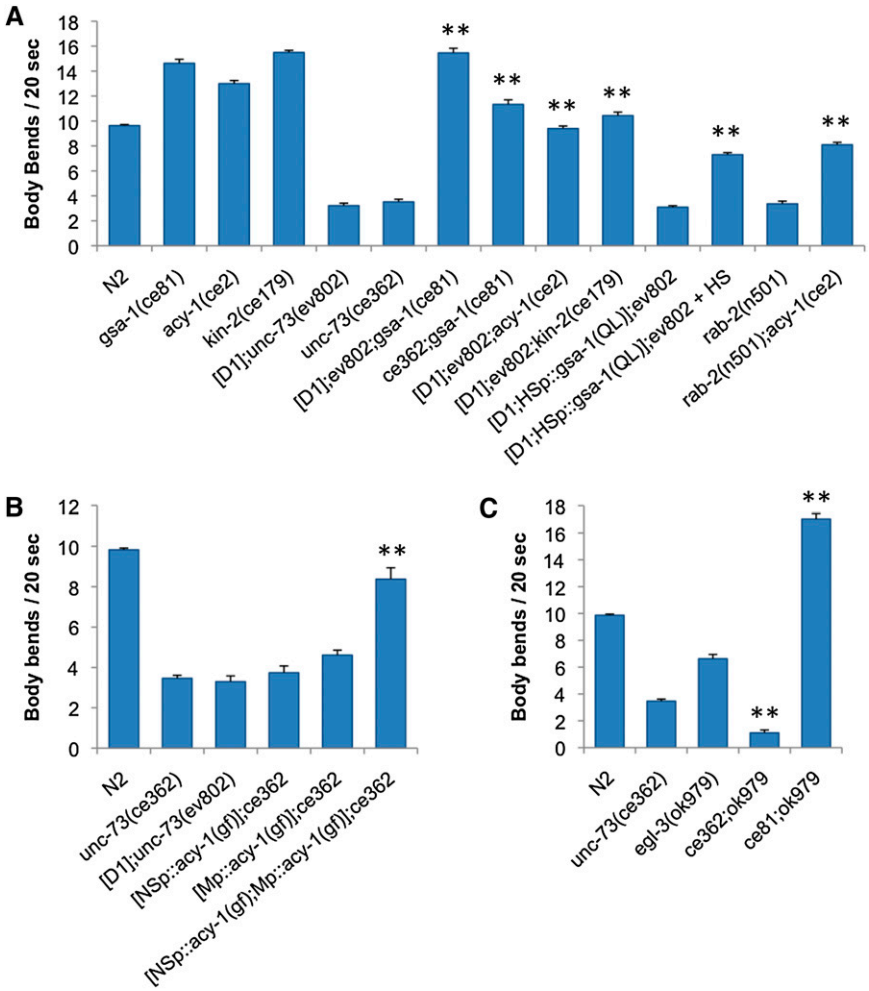

Figure 5 Constitutive $G\alpha_s$/GSA-1 pathway activity rescues *unc-73* RhoGEF-2 mutant lethargic locomotion. (A) *acy-1(ce2)* and *gsa-1(ce81)* gain-of-function mutations and the *kin-2(ce179)* loss-of-function mutation suppress *Is[D1]*; *unc-73(ev802)*, *unc-73(ce362)*, and *rab-2(n501)* locomotion defects. *gsa-1(gf)* cDNA expression in heat-shocked (HS) adult mutant animals also rescues *unc-73* RhoGEF-2 mutant lethargy. (B) Expression of an *acy-1 (gf)* cDNA exclusively in muscles (M) or the nervous system (NS) fails to rescue the *unc-73* RhoGEF-2 lethargic phenotype, although expression of the cDNAs in combination does rescue the lethargy. (C) The *unc-73(ce362)*; *egl-3(ok979)* locomotion rate is significantly lower than that of either single mutant alone. *gsa-1(ce81)* rescues the *egl-3 (ok979)* lethargic movement phenotype. Error bars show SEM. $^{**}P < 0.001$ in comparison to the corresponding *unc-73*, *rab-2*, or *egl-3* mutant using Student's *t*-test.

animals (Figure 5A) and for unknown reasons also caused a reduction in animal length (Figure 6). A *gsa-1* gain-of-function cDNA under the control of a heat-shock promoter also suppressed *Is[D1]*; *unc-73(ev802)* lethargic movement in heat-shocked adult animals (Figure 5A). These results indicate the $G\alpha_s$/GSA-1 pathway functions downstream of or in parallel to the UNC-73 RhoGEF-2 isoforms and GSA-1 activity, like that of the UNC-73 RhoGEF-2 isoforms, is not required during development, but instead functions to regulate locomotion in the mature animal.

Constitutively active $G\alpha_s$/GSA-1 pathway mutants are hypersensitive to aldicarb, indicating they release more acetylcholine into the synapse or they are more sensitive to acetylcholine (Figure 1C) (Schade *et al.* 2005). Increased acetylcholine release and sensitivity are likely factors involved in the activated $G\alpha_s$/GSA-1 pathway rescue of *unc-73* RhoGEF-2 lethargy as *gsa-1(ce81) unc-73(ce362)* and *Is [D1]*; *gsa-1(ce81) unc-73(ev802)* double mutants were hypersensitive to both aldicarb and 0.5 mM levamisole (Figure 1, C and E, and Figure S1A; data not shown). Increased acetylcholine release may be the more significant factor in aldicarb hypersensitivity since *gsa-1(ce81)* mutants are actually resistant to a lower concentration of 0.1 mM levamisole (Schade *et al.* 2005). Interestingly, levamisole sensitivity does not correlate with the locomotory phenotypes of these mutants. For example, rescued *gsa-1(ce81) unc-73(ce362)* double mutants with wild-type locomotion rates and lethargic *unc-73(ce362)* single mutants are both strongly hypersensitive to levamisole (Figures 1E and 5A). Similarly, transgenic animals that are lethargic due to reduced RHO-1 activity or hyperactive as a result of *rho-1 (gf)* expression are hypersensitive to levamisole (McMullan *et al.* 2006).

unc-73 lethargic movement is similar to that of rab-2 and unc-31

The lethargic yet coordinated locomotion phenotype of *unc-73* RhoGEF-2 mutants is separate from the severe uncoordinated movement phenotypes of genes such as *snb-1* (synaptobrevin), which are fundamental to the mechanisms of synapse function involving synaptic vesicles, and is perhaps more suggestive of a role for RhoGEF-2 activity in the modulatory mechanisms controlling locomotion rate (Figure 2) (Hall and Hedgecock 1991; Maruyama and Brenner 1991; Nonet *et al.* 1998; Steven *et al.* 2005). For example, *C. elegans* RAB2 (RAB-2) function is critical for the modulatory

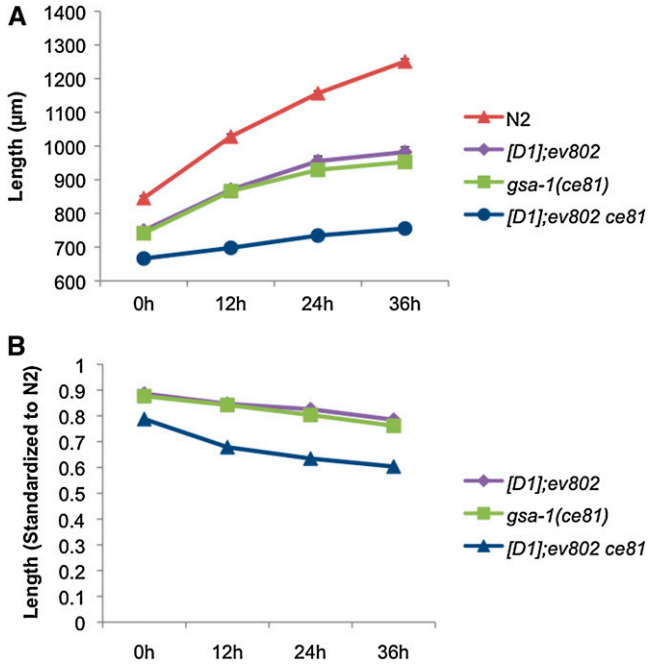

Figure 6 *unc-73* RhoGEF-2 and *gsa-1(gf)* mutants are shorter than wild type. (A) *Is[D1]*; *unc-73(ev802)* and *gsa-1(ce81)* mutants are shorter than wild type. The *Is[D1]*; *gsa-1(ce81) unc-73(ev802)* double mutants are shorter than the single-mutant animals ($P = 0.0002$; ANOVA two-factor without replication). Worm lengths were measured starting at the L4 "crescent vulva" stage and three more times every 12 hr. $n \geq 25$ for each strain. Error bars show SEM. (B) Worm lengths shown as a percentage standardized to wild type.

regulation of locomotion through its role in DCV maturation (Edwards *et al.* 2009; Sumakovic *et al.* 2009). Importantly, the *rab-2* locomotion phenotype (Park and Horvitz 1986; Chun *et al.* 2008) is very similar to *Is[D1]*; *unc-73(ev802)* and *unc-73(ce362)* slow movement phenotypes; animals are extremely slow on food, but increase their speed off food to a rate about one-third that of wild type (Figure 5A; see File S1, File S3, and File S5). We also observed that *rab-2(n501)* lethargy was suppressed by either a $G\alpha_s$/GSA-1 pathway gain-of-function mutation or treatment with phorbol ester (Figure 5A; see File S1, File S2, File S3, and File S4). Phorbol esters are stable analogs of DAG, which activate PKC and UNC-13 and can stimulate both synaptic vesicle and DCV-mediated neurotransmission (Gillis *et al.* 1996; Stevens and Sullivan 1998; Lackner *et al.* 1999; Rhee *et al.* 2002; Sieburth *et al.* 2007). The lethargy of *unc-73* RhoGEF-2 mutants was rescued by phorbol esters as well (Williams *et al.* 2007) (see File S5 and File S6). Also consistent is the fact that *unc-73* RhoGEF-2 mutants and *rab-2* mutants both were weakly resistant to aldicarb and were hypersensitive to the acetylcholine receptor agonist levamisole, suggesting similar alterations to cholinergic signaling may occur in these mutants, although Sumakovic *et al.* (2009) reported that *rab-2* mutants have wild-type levamisole sensitivity using a different method (Figure 1, A and B, and Figure S1, B and C) (Edwards *et al.* 2009). Our observations place the UNC-73 RhoGEF-2 isoforms in a category of proteins including RAB-2 and UNC-31 [*C. elegans* **c**alcium-dependent **a**ctivator **p**rotein for **s**ecretion (CAPS)], which play distinct roles in the DCV-mediated modulation of locomotion and whose very similar slow movement phenotypes are rescued by constitutive activation of the $G\alpha_s$/GSA-1 pathway (Avery *et al.* 1993; Charlie *et al.* 2006).

$G\alpha_s$/GSA-1 pathway function

Our results indicate UNC-73 RhoGEF-2 isoforms function specifically in the nervous system where they may regulate DCV-mediated transport of neuromodulators affecting locomotion. Increased GSA-1 signaling elevates DCV exocytosis from neurons, suggesting a possible explanation for $G\alpha_s$/GSA-1 pathway rescue of the *unc-73* RhoGEF-2 slow movement phenotype (Zhou *et al.* 2007). To test whether the $G\alpha_s$/GSA-1 pathway functions in neurons for rescue we expressed an ACY-1(P280S) gain-of-function protein (Schade *et al.* 2005) specifically in the nervous system of *unc-73(ce362)* mutants. Surprisingly, these animals and *ce362* animals with ACY-1(P280S) expressed specifically in the muscles remained lethargic; however, *ce362* animals containing ACY-1(P280S) in both the nervous system and in muscles were no longer lethargic (Figure 5B). Therefore, it appears $G\alpha_s$/GSA-1 pathway activation in both neurons and muscles is required to compensate for *unc-73* RhoGEF-2 neuronal defects.

UNC-73 RhoGEF-2 isoforms have functions in addition to neuropeptide signaling

EGL-3 is a proprotein convertase, which cleaves propeptides and is required for the production of functional neuropeptides (Kass *et al.* 2001). Like *unc-73* RhoGEF-2 mutants, *egl-3* mutants are resistant to aldicarb and hypersensitive to levamisole (Jacob and Kaplan 2003). Since our results indicate UNC-73 RhoGEF-2 isoforms affect the level of neuromodulatory protein in the DCVs of peptidergic neurons, we looked for evidence of a genetic interaction between *unc-73 (ce362)* and a putative *egl-3(ok979)* null mutant. The *unc-73(ce362)*; *egl-3(ok979)* double mutants move very slowly, at a rate ~95% slower than wild type, which is slower than the rate for either single mutant alone, suggesting EGL-3 and the UNC-73 RhoGEF-2 isoforms act in parallel pathways (Figure 5C). It is important to consider, however, that it is difficult to predict the effect of EGL-3 inactivation in double mutants since neuropeptides can have either positive or negative effects on locomotion and *egl-3* mutations may not affect all neuropeptides or completely inactivate them since neuropeptide staining with an FMRFamide antibody is only moderately reduced in *egl-3* mutants (Nelson *et al.* 1998; Kass *et al.* 2001; Jacob and Kaplan 2003; Husson *et al.* 2006). This makes the *unc-73*; *egl-3* phenotype difficult to interpret and may also explain why *egl-3* null mutants have such a mild locomotion phenotype with rates just 30% lower than wild type (Figure 5C). The severe lethargy of *unc-73*; *egl-3* double mutants at least suggests UNC-73 RhoGEF-2

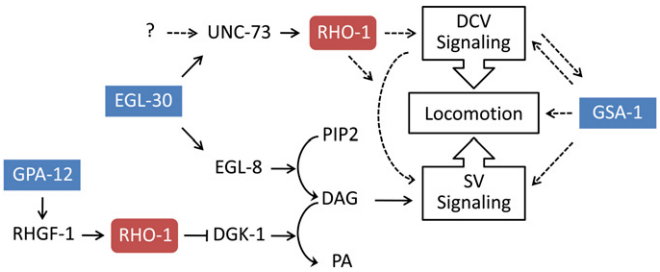

Figure 7 Rho GTPase and heterotrimeric G-protein pathway interactions regulating neurotransmission and locomotion. G-protein pathways (blue boxes) interact with Rho GTPase pathways (red boxes) directly (solid arrows) or through indirect or unknown mechanisms (dashed arrows). Rho exists in two separate populations, only one of which interacts with DGK-1 (Hiley *et al.* 2006; McMullan *et al.* 2006). The $G\alpha_o$ (GOA-1) pathway negatively regulates $G\alpha_q$ (EGL-30) and synaptic vesicle signaling, but is omitted for clarity (Hajdu-Cronin *et al.* 1999; Miller *et al.* 1999; Nurrish *et al.* 1999). UNC-73 RhoGEF-2 activity (UNC-73) functions downstream of EGL-30 and likely another factor(s) to modulate locomotion through dense core vesicle (DCV) and/or synaptic vesicle (SV) signaling. Constitutive $G\alpha_s$ (GSA-1) pathway activation can compensate for *unc-73* RhoGEF-2 mutant locomotion defects, but the mechanism is not known. See the *Discussion* and references for details (Brundage *et al.* 1996; Kozasa *et al.* 1998; Lackner *et al.* 1999; Reynolds *et al.* 2005; Schade *et al.* 2005; Charlie *et al.* 2006; Hiley *et al.* 2006; McMullan *et al.* 2006; Williams *et al.* 2007; Zhou *et al.* 2007).

isoforms may affect locomotion through molecules in addition to neuropeptides. Indeed, we observed that *unc-73* RhoGEF-2 mutations, particularly in a *gsa-1* mutant background, can influence animal length, which may result from DCV signaling defects affecting the function of proteins or growth factors in addition to neuropeptides (Figure 6).

Finally, we examined the relationship between the $G\alpha_s$/GSA-1 pathway and neuropeptide pathways affected by EGL-3 processing. In a *gsa-1(ce81)* gain-of-function background *egl-3(ok979)* lethargic movement was rescued to speeds even faster than wild type, indicating constitutive $G\alpha_s$/GSA-1 pathway activation can compensate for neuropeptide processing defects affecting locomotion (Figure 5C).

Discussion

This study examines the role of Rho GTPase pathway signaling in the regulation of *C. elegans* locomotory behavior. Mutations affecting the Rho GTPase-specific UNC-73 RhoGEF-2 isoforms result in a decreased rate of locomotion while animals continue to move in a coordinated sinusoidal manner. Our results suggest UNC-73 RhoGEF-2 isoforms affect *C. elegans* motility through changes in neurotransmission signaling involving acetylcholine and the regulation of DCV neuromodulatory protein levels in mature neurons. Cholinergic signaling alterations observed in *unc-73* RhoGEF-2 mutants treated with pharmacological agents may be due to UNC-73 RhoGEF-2 isoforms playing a direct role in cholinergic signaling mechanisms. However, phenotypic similarities between *unc-73* and DCV signaling genes and our observation of DCV signaling defects in *unc-73* mutants instead suggest a role for UNC-73 RhoGEF-2 isoforms in the DCV-mediated modulatory mechanisms regulating locomotion with observed changes in cholinergic signaling possibly a secondary effect of DCV-mediated signaling. Importantly, we observed that *unc-73* RhoGEF-2 mutant lethargy is rescued by constitutive activation of the $G\alpha_s$/GSA-1 pathway. This is consistent with the proposed role for UNC-73 RhoGEF-2 isoforms in DCV secretory pathway regulation since $G\alpha_s$/GSA-1 pathway activation increases DCV exocytosis and can bypass defects in DCV signaling (Charlie *et al.* 2006; Zhou *et al.* 2007).

unc-73, rab-2, and unc-31 have similar phenotypes

In addition to *unc-73* the genes *rab-2* and *unc-31* have very similar lethargic, yet coordinated movement phenotypes that are also rescued by $G\alpha_s$/GSA-1 pathway activation (Figure 5) (Charlie *et al.* 2006). UNC-31 is the *C. elegans* homolog of mammalian CAPS, a component of the DCV release machinery required for hormone and neuropeptide release from neuroendocrine cells (Walent *et al.* 1992; Rupnik *et al.* 2000). Although CAPS and its paralog CAPS-2 may have additional functions, including synaptic vesicle priming and catecholamine uptake into DCVs, the role of the only *C. elegans* CAPS homolog, UNC-31, specifically involves DCV docking in the process of exocytosis from neurons (Speidel *et al.* 2005; Gracheva *et al.* 2007; Jockusch *et al.* 2007; Speese *et al.* 2007; Zhou *et al.* 2007; Hammarlund *et al.* 2008). *Drosophila* CAPS function at the neuromuscular junction is also restricted to DCV exocytosis with additional cell nonautonomous effects on synaptic vesicle release believed to result from the lack of neuromodulators released from DCVs (Renden *et al.* 2001). After DCVs bud from the Golgi, the *C. elegans* RAB2 GTPase, RAB-2/UNC-108, is required for DCV maturation in a process that occurs in neuronal cell bodies upstream of DCV release mechanisms (Edwards *et al.* 2009; Sumakovic *et al.* 2009).

Thus, *unc-31*, *rab-2*, and *unc-73* RhoGEF-2 mutants not only have very similar phenotypes, including weak or no resistance to aldicarb, hypersensitivity to levamisole, and almost identical lethargic movement phenotypes that can be rescued by phorbol esters or $G\alpha_s$/GSA-1 pathway activation, but also each of the proteins encoded by these genes functions in the DCV signaling pathway (Figures 1, A and B, and 5A and Figure S1, B and C) (Avery *et al.* 1993; Miller *et al.* 1996; Daniels *et al.* 2000; Steven *et al.* 2005; Charlie *et al.* 2006; Williams *et al.* 2007; Chun *et al.* 2008; Sumakovic *et al.* 2009). The similar lethargic phenotypes of these genes are interesting in that the mutants are extremely slow on food, but they increase their speed to a rate about one-third that of wild type when removed from bacteria. It is also consistent that cholinergic motor neuron-specific *unc-73* or *rab-2* expression is not sufficient to rescue their respective locomotion phenotypes, although *unc-31* cholinergic motor neuron expression rescues *unc-31* locomotion to 80% of the wild-type rate (Figure 3) (Charlie *et al.* 2006; Chun *et al.* 2008). On the basis of these similarities we describe the *unc-*

73, *rab-2*, and *unc-31* genes as having an Lrg phenotype (**l**ethargic, but coordinated movement **r**escued by **G**α_s activation) and predict that other genes in the neuromodulatory pathways controlling locomotion will also have a Lrg phenotype.

UNC-73 RhoGEF-2 isoforms and neuromodulatory protein signaling

Decreased NLP-21::YFP neuropeptide fluorescence in axons and coelomocytes, but not the cell bodies of *unc-73* RhoGEF-2 mutants suggests at least two possible functions for UNC-73 RhoGEF-2 isoforms in the secretory pathway: (1) producing, trafficking, or maintaining the correct number of DCVs traveling down the axon and/or (2) loading or maintaining the correct number of neuromodulators in DCVs. UNC-73 RhoGEF-2 isoforms are not required for the initial production of neuropeptides since there is no difference in NLP-21::YFP cell body fluorescence in mutants compared to wild type.

UNC-73 RhoGEF-2 domain activity is specific to the GTPase Rho (Spencer *et al.* 2001). Consistent with UNC-73 RhoGEF-2 function in the secretory pathway Rho localizes to secretory granule membranes in chromaffin cells and modulates the secretory pathway in MAST cells (Price *et al.* 1995; Gasman *et al.* 1998; Ory and Gasman 2011). Another interesting finding is that ARAP1, which has both RhoGAP (**G**TPase **a**ctivating **p**rotein) and ArfGAP domains, localizes to the Golgi apparatus and can change Golgi morphology when overexpressed (Miura *et al.* 2002).

UNC-73 mammalian homologs Trio and Kalirin also function in the cellular secretory pathway. Kalirin was originally identified through its association with peptidylglycine-amidating monooxygenase (PAM), a neuropeptide processing enzyme that functions in DCVs (Alam *et al.* 1997; Mains *et al.* 1999). Kalirin and Trio modulate DCV maturation in pituitary derived AtT-20 neuroendocrine cells and their isoforms are differentially associated with Golgi, immature DCVs, and endosomes (Ferraro *et al.* 2007). Increasing Kalirin or Trio activity depletes immature DCVs of their hormone cargo, while Kalirin or Trio inhibition increases the amount of mature hormone product in mature DCVs. This role for Kalirin and Trio in the secretory pathway is mediated by their RhoGEF-1 domains, suggesting the UNC-73 RhoGEF-1 domain may have a role in the secretory pathway. Our study, on the other hand, indicates Trio and Kalirin RhoGEF-2 activity may also function in the secretory pathway.

Which secreted proteins are influenced by UNC-73 RhoGEF-2 function?

Many neuropeptides and neuropeptide receptors are known to influence muscle and neuronal activity and ultimately locomotion in *C. elegans* and other nematodes (Marks *et al.* 2001; Rogers *et al.* 2001; Keating *et al.* 2003; Husson *et al.* 2007). It is possible that one or more neuromodulators required to regulate locomotion are released from neuronal DCVs in wild-type animals but are not transported properly through the neuronal secretory pathway in *unc-73* RhoGEF-2 or *rab-2* mutants and not properly released from *unc-31* mutant neurons. The identity of this neuromodulator(s) is not known and although we use NLP-21::YFP neuropeptide to monitor movement of neuromodulatory proteins through the secretory pathway in our assays, NLP-21 is not a likely candidate since *nlp-21* RNAi does not cause locomotory defects (Kamath *et al.* 2003). In fact, a neuropeptide may not be the neuromodulator affected in *unc-73* RhoGEF-2 mutants since the severity of the locomotion defect in these mutants increases in an *egl-3* neuropeptide-processing mutant background (Figure 5C). Also, *unc-31* mutants, with compromised DCV exocytosis, are much more lethargic than *egl-3* and *egl-21* neuropeptide-processing mutants, suggesting other neuromodulatory proteins in addition to neuropeptides are regulating locomotion (Kass *et al.* 2001; Jacob and Kaplan 2003; Speese *et al.* 2007).

For example, TGFβ signaling may be affected in *unc-73* RhoGEF-2 mutants. The *C. elegans* TGFβ family member UNC-129 has a locomotory phenotype, but it is thought to result from axon guidance defects during development (Colavita *et al.* 1998). Mutations in other TGFβ signaling molecules can suppress *unc-2* mutant calcium channel lethargy, but the mechanisms are not defined (Estevez *et al.* 2004). *C. elegans* TGFβ pathways are more well known for the control of dauer formation and importantly, animal size, a trait affected in *unc-73* RhoGEF-2 mutants (Figure 6). Also relevant are observations that vertebrate TGFβ affects the activity of mature synapses at the neuromuscular junction (Chin *et al.* 2002; Fong *et al.* 2010). Although no vertebrate neurotrophin family homologs exist in *C. elegans*, it is possible other proteins regulated by UNC-73 RhoGEF-2 isoforms have neurotrophin-like functions in synaptic activity modulation (Lessmann and Brigadski 2009). Finally, biogenic amines such as serotonin and dopamine can be released from DCVs, but on the basis of phenotype analysis, our cell-specific rescue experiments, and previous double-mutant characterization these molecules do not appear to be neuromodulators affected in *unc-73* RhoGEF-2 mutants (Steven *et al.* 2005; Sudhof 2008).

Where is UNC-73 RhoGEF-2 function required to regulate locomotion?

UNC-73 RhoGEF-2 isoforms function in the nervous system to modulate *C. elegans* locomotion rate (Figure 2). Body wall muscles, which facilitate *C. elegans* locomotion, are innervated by cholinergic motor neurons, possible candidates for the specific site of UNC-73 RhoGEF-2 function within the nervous system. Indeed, we observe that neuropeptide signaling is reduced in *unc-73* RhoGEF-2 mutant cholinergic motor neurons (Figure 4) and C3-mediated Rho inactivation in these same neurons results in a slow movement phenotype, indicating UNC-73 RhoGEF-2 isoforms and the Rho pathway have roles in cholinergic motor neuron function (McMullan *et al.* 2006). However, UNC-73 RhoGEF-2

isoforms likely function in additional neurons since *unc-73* lethargy is not rescued with cholinergic motor neuron-specific expression of the UNC-73E isoform (Figure 3). Interestingly, the aldicarb resistance phenotypes of the neuropeptide processing genes *egl-3* and *egl-21* and, as mentioned previously, *rab-2* lethargy, also are not rescued by cholinergic motor neuron-specific expression (Jacob and Kaplan 2003; Chun *et al.* 2008). These observations and the rescue of *unc-73* lethargy with peptidergic *unc-73E* expression (Figure 3) are consistent with UNC-73 RhoGEF-2 isoforms, RAB-2, and the neuropeptide processing enzymes EGL-3 and EGL-21 performing a neuromodulatory function in unidentified control neurons upstream of the cholinergic motor neurons.

Gα$_s$/GSA-1 pathway activation rescues UNC-73 RhoGEF-2 mutant locomotory defects

cAMP and PKA regulate DCV and synaptic vesicle release in multiple cell types and are required for the modulation of synaptic plasticity in mammals through the modification of ion channels, the synaptic release machinery, or transcription factors. (Brandon *et al.* 1997; Seino and Shibasaki 2005). In *C. elegans*, defects in UNC-31/CAPS-mediated neuromodulator release from DCVs are suppressed by cAMP production and PKA activation downstream of Gα$_s$/GSA-1. Specifically, *unc-31* DCV docking and exocytosis defects, observed by total internal reflection fluorescence microscopy and membrane capacitance measurements, are ameliorated by both forskolin application and genetic Gα$_s$/GSA-1 pathway activation (Zhou *et al.* 2007), while *unc-31* locomotion defects are also suppressed by Gα$_s$/GSA-1 pathway activation (Charlie *et al.* 2006).

Our analysis and *rab-2* reports (Edwards *et al.* 2009; Sumakovic *et al.* 2009) suggest UNC-73 RhoGEF-2 isoforms and RAB-2 function upstream of the UNC-31–mediated mechanisms of exocytosis at the plasma membrane; therefore, one possibility is that increased Gα$_s$/GSA-1 pathway activity enlarges the readily releasable pool of DCVs at the plasma membrane to compensate for the reduced level of neuromodulator proteins sent for exocytosis in *unc-73* RhoGEF-2 and *rab-2* mutants (Zhou *et al.* 2007). Our observations with *egl-3* neuropeptide processing mutants, however, suggest the Gα$_s$/GSA-1 pathway may instead act downstream of neuromodulatory protein release from DCVs. It seems unlikely that locomotory defects resulting from unprocessed neuropeptides would be compensated for by an increased release of the unprocessed neuropeptides from DCVs, yet we observe that *egl-3* locomotion defects are rescued by Gα$_s$/GSA-1 pathway activation (Figure 5C). Another possibility is that the proposed neuromodulatory protein(s), whose secretion is affected by UNC-73, RAB-2, and UNC-31, activates a Gα$_s$/GSA-1–coupled receptor-modulating locomotion. GSA-1 activation would therefore increase acetylcholine release and increase locomotion rates in *unc-73*, *rab-2*, and *unc-31* mutants downstream of their respective DCV signaling defects. Our results further show that Gα$_s$/GSA-1 pathway activity is required in neurons and body wall muscle for complete rescue of *unc-73* RhoGEF-2 mutant lethargy (Figure 5B). This is consistent with the role of the Gα$_s$/GSA-1 pathway in *C. elegans*, which functions in both the nervous system and body wall muscles to modulate the rate of locomotion (Reynolds *et al.* 2005).

Although our data are consistent with the above hypothesis, there is no definitive proof the Gα$_s$/GSA-1 pathway functions downstream of DCV signaling to regulate *C. elegans* locomotion. Gα$_s$/GSA-1 pathway activation increases acetylcholine release, which could rescue *unc-73* RhoGEF-2 mutant lethargy whether UNC-73 functions in either a DCV or a synaptic vesicle signaling pathway (Figure 1C and Figure S1A) (Schade *et al.* 2005). However, aldicarb-hypersensitive *dgk-1* mutants do not rescue the *unc-73* RhoGEF-2 lethargic movement phenotype, indicating not all mutants that increase acetylcholine release can rescue *unc-73* lethargy (Miller *et al.* 1999; Steven *et al.* 2005).

UNC-73 RhoGEF-2 isoforms have Gα$_q$/EGL-30–independent functions

Screens for mutants that suppress the hyperactive and slow growth phenotypes of an overactive Gα$_q$/EGL-30 pathway identified UNC-73E as a direct downstream effector of Gα$_q$/EGL-30 acting in parallel to its well-known target phospholipase C$_\beta$/EGL-8 (Figure 7) (Williams *et al.* 2007). Several observations lead us to believe UNC-73 RhoGEF-2 isoforms also have Gα$_q$/EGL-30–independent functions in the regulation of locomotion: (1) *unc-73* RhoGEF-2 mutants have weak resistance to the acetylcholinesterase inhibitor aldicarb and are hypersensitive to levamisole, while *egl-30* mutants display strong aldicarb resistance and are no different from wild type on levamisole (Figure 1) (Lackner *et al.* 1999; Williams *et al.* 2007); (2) EGL-30 modulates locomotion through its activity in cholinergic motor neurons (Lackner *et al.* 1999), but UNC-73 RhoGEF-2 isoform effects on locomotion involve neurons in addition to cholinergic motor neurons (Figure 3); and (3) genetic activation of the Gα$_s$/GSA-1 pathway completely rescues *unc-73* RhoGEF-2 mutant locomotory defects (Figure 5A), but the same Gα$_s$/GSA-1 pathway activation does not even partially rescue *egl-30* locomotory defects (Reynolds *et al.* 2005). UNC-73E is likely a direct downstream Gα$_q$/EGL-30 effector with regard to egg laying and growth (Williams *et al.* 2007); however, the relationship between EGL-30 and UNC-73E in the modulation of locomotion is not as clear and may involve one or more additional factors upstream of UNC-73 ("?" in Figure 7).

In conclusion, our analysis suggests UNC-73 RhoGEF-2 isoforms regulate locomotion through changes in neurotransmitter signaling. We propose that the UNC-73 RhoGEF-2 isoforms are required for DCV-mediated neuromodulatory protein signaling that regulates the rate of locomotion upstream of the Gα$_s$/GSA-1 pathway in *C. elegans*. Further experiments are required to identify the specific connections between the Rho GTPase and Gα$_s$/GSA-1 pathways and DCV signaling.

Acknowledgments

We thank Josh Kaplan, Ken Miller, Queelim Ch'ng, and the *Caenorhabditis* Genetics Center [which is funded by the National Institutes of Health (NIH) National Center for Research Resources] for nematode strains; Bruce Bamber, Andy Fire, Josh Kaplan, Ken Miller, Stephen Nurrish, Dave Pilgrim, Jim Rand, Piali Sengupta, and Derek Sieburth for plasmids; Qing Zhao for assistance with statistical analysis; and Rick Komuniecki, Gareth Harris, Thuy Tran, and the reviewers for helpful comments that improved the manuscript. This work was supported by NIH grant 1R15NS062406 (to R.M.S.).

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Communicating editor: D. I. Greenstein

GENETICS

Supporting Information
http://www.genetics.org/content/suppl/2011/07/13/genetics.111.131227.DC2

UNC-73/Trio RhoGEF-2 Activity Modulates *Caenorhabditis elegans* Motility Through Changes in Neurotransmitter Signaling Upstream of the GSA-1/Gα_s Pathway

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DOI: 10.1534/genetics.111.131227

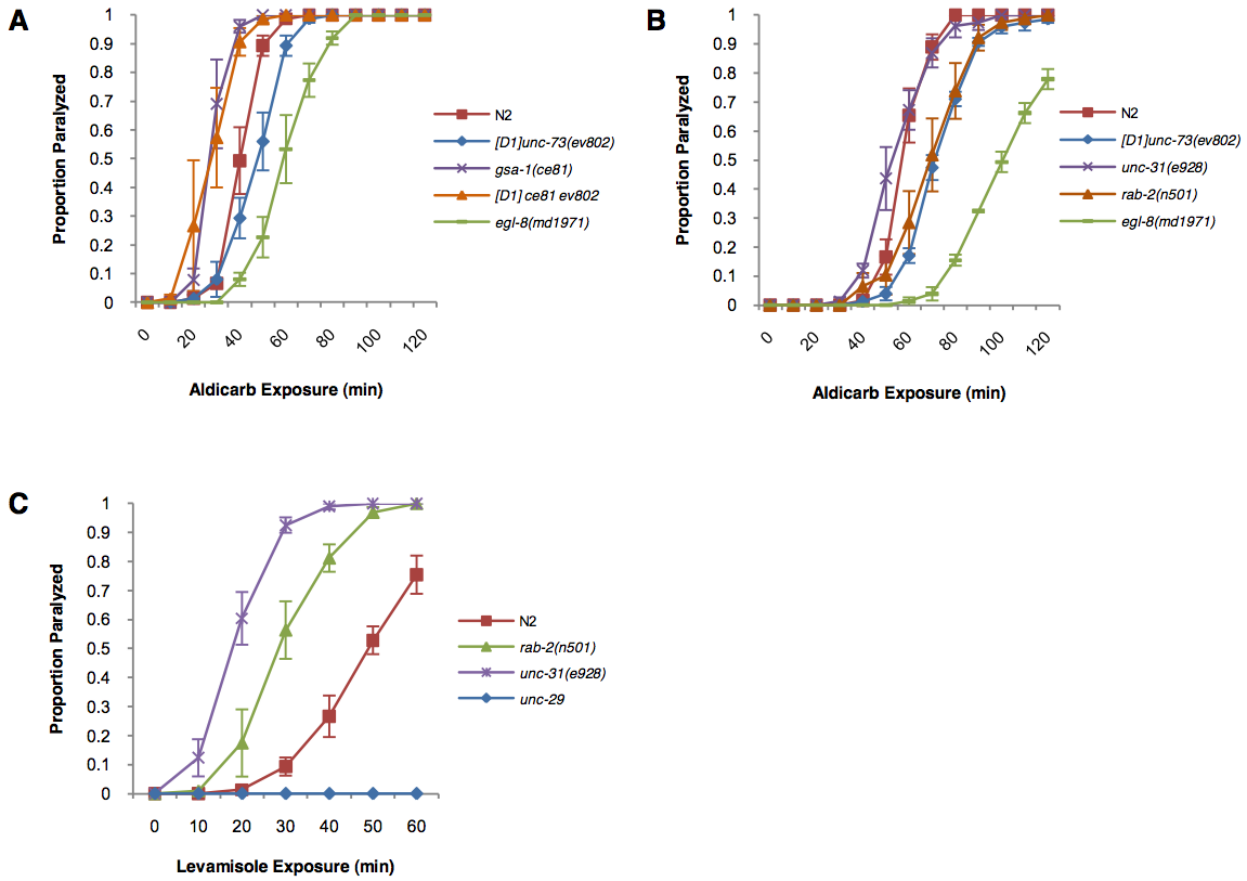

Figure S1 *rab-2*, *unc-31* and *unc-73* RhoGEF-2 mutants have altered neurotransmitter signaling. (A and B) Animals were examined for paralysis on NGM plates containing 1 mM aldicarb, an acetylcholinesterase inhibitor. *rab-2* and *unc-73* RhoGEF-2 mutants are weakly resistant to aldicarb compared to wild-type N2 animals and moderately resistant *egl-8* animals. The *unc-31* response to aldicarb is similar to N2. Gain-of-function *gsa-1(ce81)* mutants and *gsa-1(ce81) unc-73(ce362)* double mutants are hypersensitive to aldicarb. (C) Animals were examined for paralysis on NGM plates containing 0.5 mM levamisole, an acetylcholine receptor agonist. *rab-2* and *unc-31* mutants are hypersensitive to levamisole compared to N2. Control *unc-29* levamisole-sensitive acetylcholine receptor subunit mutants are completely resistant to 0.5 mM levamisole. Error bars indicate SEM.

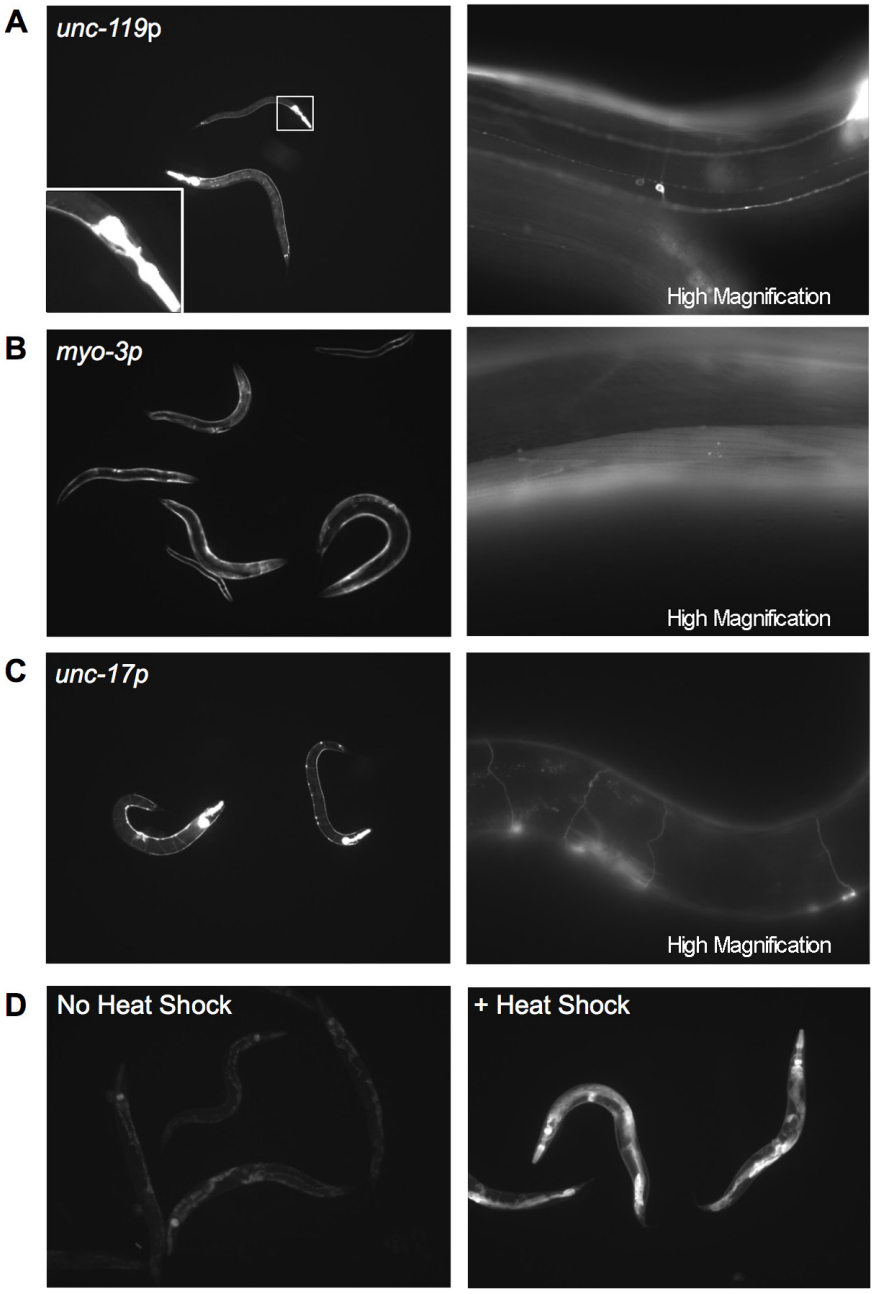

Figure S2 Cell-specific and temporally restricted expression of UNC-73E::GFP in young adult *unc-73(ev802)* animals. (A) *[D1; unc-119p::unc-73e::gfp] ev802* animals express UNC-73E::GFP in all neurons. The nerve ring and ventral cord are visible under low magnification (left panel; inset). Individual neurons within the midbody of a representative animal are visible under higher magnification (right panel). These animals have wild-type rates of movement (Fig 2). (B) *[D1; myo-3p::unc-73e::gfp] ev802* animals express UNC-73E::GFP in body wall and vulval muscle cells (left panel). The striations within a single body wall muscle cell of a representative animal are visible under higher magnification (right panel). These animals are lethargic (Fig 2). (C) *[D1; unc-17p::unc-73e::gfp] ev802* animals express UNC-73E::GFP in cholinergic motor neurons (left panel). The commissural axons of these neurons are visible at higher magnification in a representative animal. These animals are lethargic (Fig 2). (D) Heat shock of *[D1; hsp-16p::unc-73e::gfp] ev802* adult animals induces the expression of UNC-73E::GFP. *[D1; hsp-16p::unc-73e::gfp] ev802* adult animals were incubated for 40 minutes at 33° to induce the production of UNC-73E::GFP (+ Heat Shock). Heat shocked animals have wild-type rates of locomotion (Fig. 2). Sibling adult animals that were not heat shocked (No Heat Shock) only weakly express UNC-73::GFP in the pharynx and remain lethargic (Fig. 2). Intestinal autofluorescence is also visible. Both pictures in D were taken with the same exposure setting. The pharynx expression visible in A and C is likely due to recombination between the injected constructs in these strains, as described in Steven *et al*. (2005).

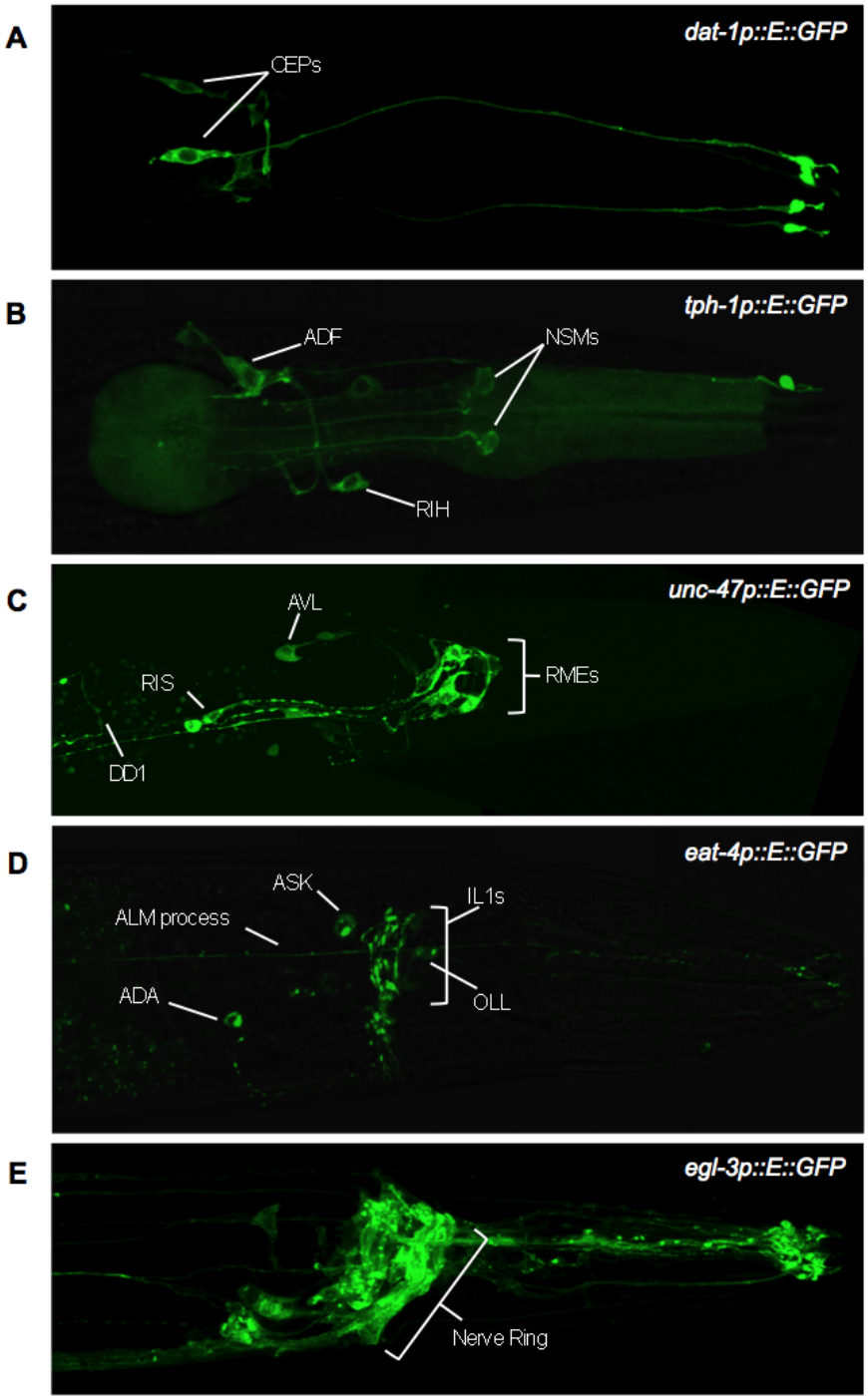

Figure S3 Cell-specific expression of UNC-73E::GFP in *unc-73(ev802)* animals. All images show the same head region of the animal with anterior to the right and ventral down (except in B and C). Neurons identified by morphology and position are labeled. (A) The *dat-1* promoter drives UNC-73E::GFP expression in all of the dopaminergic neurons including four CEP neurons in the head. (B) The *tph-1* promoter drives expression in the serotonergic neurons including the ADFs and NSMs in the head region and rarely in the RIH. Dorsal view. (C) A ventral view of UNC-73E::GFP expression controlled by the *unc-47* promoter. GABAergic neurons including four RMEs, the AVL, RIS and the DD1 motor neuron commissure are visible in the head. (D) The *eat-4* promoter drives expression in glutamatergic neurons including the ADA, ASK, OLL, IL1s and the ALM axon. (E) The *egl-3* promoter drives expression in peptidergic neurons. The nerve ring is indicated.

Files S1-S6

Files S1-S6 are available for download as .mov files at .

File S1

Video of N2 animals on control plates.

Animals move freely on control plates containing the ethanol carrier, but no phorbol ester (PMA). Animals will occasionally pause, but they do not tend to coil or reverse with high frequency.

File S2

Video of N2 animals on PMA plates.

Animals are more active on plates containing PMA. Animals rarely appear still and they tend to coil and reverse with high frequency.

File S3

Video of *rab-2(n501)* animals on control plates.

Animals move very slowly on control plates containing the ethanol carrier, but no PMA. Animals spend most of the time making small foraging head movements and show a low tendency to coil or reverse.

File S4

Video of *rab-2(n501)* animals on PMA plates.

Animals are more active on plates containing PMA. Animals rarely appear still and they tend to coil and reverse with high frequency.

File S5

Video of *unc-73(ce362)* animals on control plates.

Animals move very slowly on control plates containing the ethanol carrier, but no PMA. Animals spend most of the time making small foraging head movements and show a low tendency to coil or reverse.

File S6

Video of *unc-73(ce362)* animals on PMA plates.

Animals are more active on plates containing PMA. Animals rarely appear still and they tend to coil and reverse with high frequency. There is an example of one animal in the video that is “stuck” in a tightly coiled position. Animals can remain in this position for several seconds and N2 and *rab-2(n501)* animals treated with PMA can also be observed in this state.