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Evolution of pharyngeal behaviors and neuronal functions in free-living soil nematodes

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Summary

To explore the use of *Caenorhabditis elegans* and related nematodes for studying behavioral evolution, we conducted a comparative study of pharyngeal behaviors and neuronal regulation in free-living soil nematodes. The pharynx is divided into three parts: corpus, isthmus and terminal bulb, and pharyngeal behaviors consist of stereotyped patterns of two motions: pumping and peristalsis. Based on an outgroup species, *Teratocephalus lirellus*, the ancestral pattern of pharyngeal behaviors consisted of corpus pumping, isthmus peristalsis and terminal bulb pumping, each occurring independently. Whereas corpus pumping remained largely conserved, isthmus and terminal bulb behaviors evolved extensively from the ancestral pattern in the four major free-living soil nematode families. In the Rhabditidae family, which includes *Caenorhabditis elegans*, the anterior isthmus switched from peristalsis to pumping, and anterior isthmus and terminal bulb pumping became coupled to corpus pumping. In the Diplogasteridae family, the terminal bulb switched from pumping to peristalsis, and isthmus and terminal bulb became coupled for peristalsis. In the Cephalobidae family, isthmus peristalsis and terminal bulb pumping became coupled. And in the Panagrolaimidae family, the posterior isthmus switched from peristalsis to pumping. Along with these behavioral changes, we also found differences in the neuronal regulation of isthmus and terminal bulb behaviors. M2, a neuron that has no detectable function in *C. elegans*, stimulated anterior isthmus peristalsis in the Panagrolaimidae. Further, M4 was an important excitatory neuron in each family, but its exact downstream function varied between stimulation of posterior isthmus peristalsis in the Rhabditidae, isthmus/terminal bulb peristalsis in the Diplogasteridae, isthmus peristalsis and terminal bulb pumping in the Cephalobidae, and posterior isthmus/terminal bulb pumping in the Panagrolaimidae. In the Rhabditidae family, although M4 normally has no effect on the terminal bulb, we found that M4 can stimulate the terminal bulb in *C. elegans* if the Ca^{2+}-activated K^+ channel SLO-1 is inactivated. *C. elegans slo-1* mutants have generally increased neurotransmission, and in *slo-1* mutants we found novel electropharyngeogram signals and increased pumping rates that suggested activation of M4-terminal bulb synapses. Thus, we suggest that the lack of M4-terminal bulb stimulations in *C. elegans* and the Rhabditidae family evolved by changes in synaptic transmission. Altogether, we found behavioral and neuronal differences in the isthmus and terminal bulb of free-living soil nematodes, and we examined potential underlying mechanisms of one aspect of M4 evolution. Our results suggest the utility of *Caenorhabditis elegans* and related nematodes for studying behavioral evolution.

Supplementary material available online at
http://jeb.biologists.org/cgi/content/full/209/10/1859/DC1

Key words: evolution, nematode, *Caenorhabditis elegans*, behavior, neuronal function.

Introduction

To adapt to different environments, animals have evolved differences in their behaviors. The model organism *Caenorhabditis elegans* is a simple nematode that displays reproducible and easily observed behaviors (e.g. feeding, chemotaxis, egg-laying), whose neuronal and molecular control has been extensively analyzed (Riddle et al., 1997). Using these well-studied *C. elegans* behavioral paradigms as starting points, comparative studies of evolutionary differences in other nematode species may provide useful models for studying the mechanisms by which behaviors evolve. Comparative studies between *C. elegans* and other nematodes have been previously conducted. However, they often focused on evolutionary similarities, with the aim of demonstrating the

applicability of *C. elegans* knowledge towards understanding other nematodes, and *vice versa*. For example, studies in the parasite *Ascaris* focused on anatomical and functional similarities to *C. elegans* in the ventral cord locomotory neurons (Johnson and Stretton, 1980; Stretton et al., 1978; Walrond et al., 1985), and studies in the parasite *Strongyloides* focused on similarities to *C. elegans* in the control of chemotaxis by amphid sensory neurons (Ashton et al., 1999; Forbes et al., 2004). We were interested instead in investigating nematode behaviors and their neuronal regulation explicitly for their evolutionary differences.

Nematodes feed using a neuromuscular organ in the head termed the pharynx. Pharyngeal behaviors in *C. elegans* consist of well-defined, stereotyped patterns of muscle contractions, which are regulated by a small, self-contained pharyngeal nervous system. The neuronal and molecular bases of pharyngeal feeding behaviors in *C. elegans* have been well characterized (Avery and Thomas, 1997), but in other nematode species, they are not as well understood. In particular, *C. elegans* is a free-living soil nematode (nematodes can be classified ecologically as free-living or parasitic, and soil-dwelling or aquatic). Characterization of pharyngeal behaviors in other free-living soil nematodes has been rather limited, and generally did not examine the regulatory functions of pharyngeal neurons (Doncaster, 1962; Mapes, 1965; von Lieven, 2003).

Thus, as a model for studying behavioral evolution in *C. elegans* and related nematodes, we examined how pharyngeal behaviors and their neuronal regulation evolved in free-living soil nematodes. Four main families constitute the large majority of free-living soil nematode species: the Rhabditidae (which includes *C. elegans*), the Diplogasteridae, the Cephalobidae, and the Panagrolaimidae. We began with direct observations of pharyngeal behaviors from these four families, to obtain a systematic model of how their pharyngeal behaviors evolved. Then, to understand what changes in neuronal function corresponded with the behavioral differences, we used laser ablations (Bargmann and Avery, 1995) to define the functions of homologous pharyngeal neurons. And finally, we analyzed a *C. elegans* mutant, whose phenotypes suggested that changes in synaptic strength may have contributed to the functional evolution of a specific pharyngeal neuron. Overall, our study provides a basic, broad characterization of evolutionary differences in pharyngeal behaviors and neuronal functions in free-living soil nematodes, and demonstrates the potential utility of using *C. elegans* and related nematodes as a system for studying behavioral evolution.

Materials and methods

Strains

Free-living soil nematode species used in this study include: in family Panagrolaimidae: *Panagrolaimus sp.* PS1159, *Panagrolaimus sp.* PS1732 and *Panagrellus redivivus* DA1711 (derived by 28× sibling inbreeding starting from strain PS1163); in family Cephalobidae: *Cephalobus sp.* DWF1301, *Acrobeloides sp.* PS1146, *Cephalobus cubaensis* PS1197; in family Diplogasteridae: *Pristionchus pacificus* PS312, *Aduncospiculum halicti* JB120; in family Rhabditidae: *C. elegans* N2, *Oscheius myriophila* DF5020, *Rhabditis blumi* DF5010, *Mesorhabditis longespiculosa* DF5017, *Teratorhabditis palmarum* DF5019, *Pelodera strongyloides* DF5013, *Poikilolaimus regenfussi* SB199.

In addition to the above species, we also used *Teratocephalus lirellus* PDL0011 from the family Teratocephalidae, as an outgroup species. An outgroup species is a species outside of the group of interest (i.e. free-living soil nematodes, in this study) used to determine the evolutionary polarity of specific characters within the group of interest. That is, assuming the principle of parsimony, a character in the group of interest is either ancestral or derived, based on whether the character is also present or absent in the outgroup species, respectively (Ridley, 1996). Usually, close relatives of the group of interest are selected as outgroup species. We chose *T. lirellus* because of its close relation to the free-living soil nematodes (Blaxter et al., 1998), and also because *T. lirellus* had previously been used as an outgroup to the free-living soil nematodes (Zhang and Baldwin, 2001).

Of the above strains, *T. lirellus* PDL0011 was maintained at 19°C on soil agar plates consisting of 1% agar, 1% soil and 5 μg ml^{-1} cholesterol (De Ley and Mundo-Ocampo, 2004), and seeded with *E. coli* HB101 (Boyer and Roulland-Dussoix, 1969). All other strains were maintained at 19°C on NGMSR plates (Davis et al., 1995) seeded with *E. coli* HB101, except DWF1301, PS1146, PS1197, DF5017 and DF5019, which were maintained at 24°C.

For *C. elegans* experiments, we used the wild-type strain N2, as well as mutant strains that contained the following alleles: *slo-1(js379)* (Wang et al., 2001), *eat-2(ad465)* (McKay et al., 2004), *eat-4(ky5)* (Lee et al., 1999) and *eat-5(ad1402)*, which is a putatively null deletion (835–2274 bp of F13G3.8) that removes exons 2 through 4. All *C. elegans* strains were maintained at 19°C on NGMSR plates seeded with *E. coli* HB101.

Visual observations of pharyngeal behaviors in free-living soil nematode species

Using video microscopy, we observed free-living soil nematode species for differences in pharyngeal behaviors. Bacterial suspensions were made by scraping *E. coli* from 1–2 seeded HB101 plates into 100 μl of M9 medium followed by thorough mixing. Animals were then transferred to 2.5 μl of the bacterial suspension, placed either on an unseeded NGMSR plate or a ~1 mm thick NGMSR agar pad, and coverslips placed on top. We typically waited ~30–60 min before making observations because feeding motions were often erratic and inconsistent immediately after application of the coverslip, presumably because the animals were disturbed by the protocol (this problem was more pronounced in some species). Pharyngeal motions were then observed using a Zeiss Axiophot microscope with a 40×, 63× or 100× objective. Videos were taken with a Hitachi kP-160 CCD camera and digitized using Adobe Premiere v6.5. Compression of videos

into MPEG files was done *via* SmartVideoConverter v1.5.15 (DoEase Software). Adults and larvae of each species generally displayed similar pharyngeal behaviors, and movies provided as supplementary material were typically taken from larvae, since the smaller larvae permitted easier focusing and tracking for video recordings.

Laser ablations in free-living soil nematodes

We chose *P. pacificus* PS312, *Cephalobus sp.* DWF1301 and *Panagrolaimus sp.* PS1159 as representatives of the Diplogasteridae, Cephalobidae and Panagrolaimidae families, respectively, for laser ablations because they have large broods, are easily anesthetized, and are easy to handle. Laser ablations were performed on newly hatched larvae less than 4 h old as previously described for *C. elegans* (Bargmann and Avery, 1995), except for the anesthetic conditions. For PS312, the larvae were anesthetized on 10 mmol l^{-1} NaN_3 pads, as in *C. elegans*. For PS1159, the larvae were anesthetized on 20 mmol l^{-1} NaN_3 pads. For DWF1301, larvae were placed in ~1 μl of 100 mmol l^{-1} NaN_3 on top of 10 mmol l^{-1} NaN_3 pads for ~30 s until the animals relaxed by curling up, followed by application of the coverslip. We assayed pumping and peristalsis rates of operated animals after they reached adulthood, determined by the presence of laid eggs on the plate. The animals were observed by microscopy as described above, and their pumping and peristalsis rates were determined by counting the number of pumps/peristalsis in 1 min. For MC ablations (see Results), the Panagrolaimidae species *P. redivivus* DA1711 was also used, but MC could not be distinguished from I2 in DA1711 so both nuclei types had to be killed. *P. redivivus* DA1711 was anesthetized on 10 mmol l^{-1} NaN_3 pads, and adulthood was determined by the presence of oocytes in females or spicules in males.

Two types of controls were performed to verify that the changes in pharyngeal function were specific to the neuron ablated. We performed (1) mock ablations, where the larvae were anesthetized but no neurons were ablated, to control for nonspecific effects of anesthetization, and (2) nearby ablations, where nearby neurons were ablated (e.g. M3 or NSM for M4), to control for nonspecific effects of laser damage. In general, the effects of mock and nearby ablations were indistinguishable, and the control results, presented in the figures were derived from mock ablations.

In the outgroup species, *T. lirellus*, culture difficulties prevented laser ablations from being performed. Culture of *T. lirellus* was generally challenging due to long generation times and sensitivity to growth conditions, and single animal cultures, which are important for laser ablation experiments, were invariably lost (the animals either died or could not be found on the plates).

Food density dependence assays

Normal bacterial suspensions were prepared as above (i.e. 1–2 HB101 plates/100 μl M9), whereas dilute bacterial suspensions were prepared by diluting the normal suspensions 50-fold for *Cephalobus sp.* DWF1301 and *Pristionchus pacificus* PS312, or 20-fold for *Panagrolaimus sp.* PS1159 (PS1159 fed inconsistently or not at all when placed in 50-fold or more dilute suspensions). We then placed coverslips over each animal in both normal and dilute bacterial suspensions, and counted their pumping and peristalsis rates.

PS1159 and DWF1301 animals were often hyperactive and hard to track under the microscope in the dilute condition. Roaming behaviors in *C. elegans* can be reduced if they are grown on poor quality food (B. Shtonda and L. Avery, manuscript submitted for publication), such as the *E. coli* strain DA837 (Davis et al., 1995). We decreased the hyperactive motions of PS1159 and DWF1301 animals in the dilute condition by placing them on DA837 plates for one night prior to the assay. Both the normal and dilute pumping/peristalsis rates reported in the food density dependence experiments for PS1159 and DWF1301 animals are from DA837 preconditioned animals, to ensure consistency in the comparisons. Pumping/peristalsis rates at normal food density in preconditioned PS1159 and DWF1301 animals are similar to those of unconditioned animals, indicating that DA837 preconditioning does not significantly affect pumping/peristalsis rates, at least at normal food density. For PS312, DA837 preconditioning was not necessary.

Transgenic C. elegans

We made *Pceh-28p::SNB-1::GFP* constructs by overlap extension PCR (Hobert, 2002). We amplified *Pceh-28*, 2.4 kb of sequence upstream of the *ceh-28* start ATG, from genomic DNA, and *SNB-1::GFP* from pSB120.65 (courtesy of M. Nonet). Then we fused the two together by additional PCR. The resulting products were coinjected with either the pRAK3 [*rol-6(d)*] plasmid (Davis et al., 1995) or *Podc-1::GFP* (PCR product), which expresses in intestinal cells (J. T. A. Chiang, unpublished observations), as transformation markers. We made *Podc-1::GFP* by amplifying *Podc-1*, 3.0kb sequence upstream of the start ATG of *odc-1* (Macrae et al., 1995), from genomic DNA, and *GFP* from pPD95.75 (A. Fire, Fire Lab Vector Kit–June 1995, ftp://www.ciwemb.edu/pub/FireLabInfo/FireLabVectors/1995_Vector_Kit/Vec95_Docs/Vec95Doc.rtf.), then fusing the two by additional PCR. Injections for transformation were performed following standard procedures (Mello and Fire, 1995). Pictures were taken on a Zeiss Axiophot microscope using a MaxCam CM7-2E CCD camera (Finger Lakes Instrumentation, Lima, NY, USA).

Two independent *Pslo-1::SLO-1::GFP* lines were gifts from the lab of Michael Nonet. They were generated by the coinjection of *Pslo-1::SLO-1::GFP* and pJM23 (lin-15+) into *unc-64(e246); slo-1(js100); lin-15(n765ts)* mutants. *Pslo-1::SLO-1::GFP* is a fusion of a 5.2 kb *Pslo-1* promoter with SLO-1 and GFP cDNAs. Pictures were taken as described above.

Electropharyngeograms

Electropharyngeograms (EPGs) on exposed pharynxes bathed in Dent's saline and 1 μmol l^{-1} serotonin (5HT) were

performed as previously described (Davis et al., 1995), with one exception. Although novel single positive spikes in *slo-1* mutant EPGs (see Results) were detectable using standard Dent's saline containing 3.0 mmol l^{-1} Ca^{2+}, those single positive spikes were larger in amplitude and easier to distinguish when we increased [Ca^{2+}] to 5.0 mmol l^{-1}. Therefore, all results presented were derived from EPGs using modified Dent's containing 5.0 mmol l^{-1} [Ca^{2+}], although the results were qualitatively similar when using standard Dent's saline. EPG currents were digitally sampled and recorded as previously described (Davis et al., 1995). Digitized EPG recordings were then viewed and analyzed using Igor Pro 4.05 from Wave Metrics (Lake Oswego, OR, USA). Quantification of single positive spikes on EPGs was assisted by Igor Pro procedures (available upon request), which scanned for positive spikes that are not followed by full pharyngeal action potentials.

Laser ablations in slo-1 mutants

MC and M3 ablations in wild-type and *slo-1* mutants were performed using standard procedures (Bargmann and Avery, 1995). Mock, M4, and M5 laser ablations in *eat-2; eat-4; slo-1* animals were also performed using standard procedures, except that all animals were subsequently grown on HB101 seeded plates containing 5 mmol l^{-1} arecoline. We added arecoline because M4 ablation normally causes larval arrest due to the loss of isthmus peristalsis (Avery and Horvitz, 1987), but growth of M4 ablated worms can be improved with 5 mmol l^{-1} arecoline in the culture plates (in M4– animals, 10% grow to adulthood on HB101 plates, whereas 70% reach adulthood on HB101+5 mmol l^{-1} arecoline plates; L. Avery, unpublished observations). The mechanism by which arecoline improves growth of M4– animals is unknown; it may be *via* general depolarization of the pharyngeal membrane, as that is the mechanism by which *eat-6* improves growth of M4– animals (Davis et al., 1995). Importantly, EPGs of mock ablated *eat-2; eat-4; slo-1* animals grown on 5 mmol l^{-1} arecoline were similar to EPGs of animals grown under standard conditions, indicating that growth on 5 mmol l^{-1} arecoline had no major effects on EPGs. In particular, single positive spike rates between *eat-2; eat-4; slo-1* animals grown on standard conditions were indistinguishable from *eat-2; eat-4; slo-1* animals grown on 5 mmol l^{-1} arecoline.

d-tubocurarine studies

For the 100 μmol l^{-1} d-tubocurarine studies, EPGs were recorded for 2 min in 100 μl of 5.0 mmol l^{-1} [Ca^{2+}] Dent's+5HT as described above, followed by the addition of another 100 μl of either the identical saline (control) or the same saline with 200 μmol l^{-1} d-tubocurarine added (experiment), to achieve a final concentration of 100 μmol l^{-1} d-tubocurarine. Single positive spike rates before and after drug addition were analyzed as described. Statistical significance between d-tubocurarine and control treatments was tested on differences in single positive spike rates (i.e. after treatment minus before treatment) in individual animals.

Pumping rate assays of slo-1 mutants

We examined *eat-5* and *eat-5; slo-1* pumping rates in L1 larvae selected within 6 h of hatching because the *eat-5* phenotype of electrically isolated terminal bulb (TB) is virtually 100% penetrant in larvae, but significantly less so in adults (Starich et al., 1996). We measured *eat-5* and *eat-5; slo-1* TB pumping rates by counting for 1 min at 19°C under a Zeiss Axiophot microscope using a 20× objective.

Results

Background – Pharyngeal behaviors and neuronal functions in C. elegans

The *C. elegans* pharynx is divided into three muscle groups (Fig. 1A): the corpus, the isthmus and the terminal bulb (Albertson and Thomson, 1976; Avery and Thomas, 1997). Pharyngeal behaviors consist of stereotyped patterns of two motions, termed 'pumping' and 'peristalsis'. Pumps are simultaneous contractions of entire muscle groups that open the pharyngeal lumen, and peristalses are posteriorly sweeping contraction waves (Avery and Horvitz, 1987; Avery and Horvitz, 1989; Avery and Thomas, 1997). Anteriorly in the *C. elegans* pharynx, pumping occurs in the corpus and anterior isthmus to suck in bacteria, and posteriorly, pumping occurs in the terminal bulb to break up ingested bacteria with the grinder (Fig. 1B). Peristalsis occurs only in the posterior isthmus, and transports food between the two regions of pumping (Fig. 1B). Corpus, anterior isthmus and terminal bulb pumping are coupled in *C. elegans* due to gap junctions (see below), such that all three regions contract together with each pump (Fig. 1B). In contrast, posterior isthmus peristalsis occurs separately from pumping.

Pharyngeal behaviors in *C. elegans* are regulated by a pharyngeal nervous system of 14 neuronal types (Albertson and Thomson, 1976). Interestingly, only a small subset of these neurons is known to have readily observable functions. Specifically, only two are known to be major excitatory neurons in *C. elegans* (schematized in Fig. 1C): M4, which is required for posterior isthmus peristalsis (Avery and Horvitz, 1987), and MC, which stimulates pumping (Avery and Horvitz, 1989; Raizen et al., 1995). The corpus muscles are stimulated to pump *via* direct MC innervations (McKay et al., 2004; Raizen et al., 1995), whereas anterior isthmus and TB pumping are indirectly stimulated *via* gap junctions between the corpus and isthmus, and isthmus and terminal bulb. Each time MC stimulates the corpus, the excitations spread rapidly to the anterior isthmus and terminal bulb and cause the anterior isthmus and terminal bulb muscles to pump simultaneously with the corpus (Starich et al., 1996; M. Steciuk, unpublished observations).

The isthmus and terminal bulb evolved differences in their spatial and coupling patterns of pumping and peristalsis

Using video microscopy, we examined pharyngeal behaviors in a broad sample of free-living soil nematode species from each of the four major families (see Materials

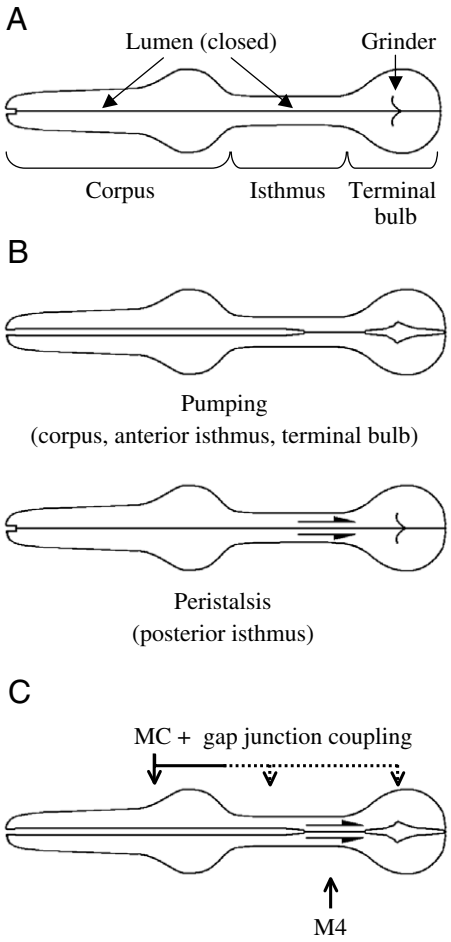


Fig. 1. Pharyngeal anatomy, behaviors, and neuronal stimulation in *C. elegans.* (A) The three muscle groups in the *C. elegans* pharynx. The corpus is large and anterior, the isthmus is narrow and in the middle, and the terminal bulb most posterior. There is a grinder in the terminal bulb for chewing bacteria. The lumen at rest is closed as shown. (B) Pumps are simultaneous contractions of entire muscle groups that open the pharyngeal lumen. Pumping occurs in the corpus, anterior isthmus, and terminal bulb in *C. elegans* (indicated by the open lumen). Peristalses are posteriorly moving contraction waves, and occur in the posterior isthmus in *C. elegans* (indicated by the arrows). (C) Stimulation of pumping and peristalsis by the two major excitatory pharyngeal neuron types in *C. elegans*, M4 and MC. M4 is required for posterior isthmus peristalsis. MC innervates the corpus and directly stimulates corpus pumping, which indirectly and simultaneously stimulates the anterior isthmus and terminal bulb to pump, since the anterior isthmus and terminal bulb are electrically coupled to the corpus *via* gap junctions.

and methods for the complete list). Additionally, to help determine the ancestral pattern of pharyngeal behaviors, we observed a close relative, *Teratocephalus lirellus*, as the outgroup species (Blaxter et al., 1998). Anatomically, the pharynxes in all of these species were similarly divided into the corpus, isthmus and terminal bulb (albeit with minor anatomical variations; see Chitwood and Chitwood, 1974; Maggenti, 1981; von Lieven, 2003; von Lieven and Sudhaus, 2000; Zhang and Baldwin, 1999). Yet behaviorally, each family, as well as *T. lirellus*, exhibited significant differences. Species within the same families had largely similar behaviors.

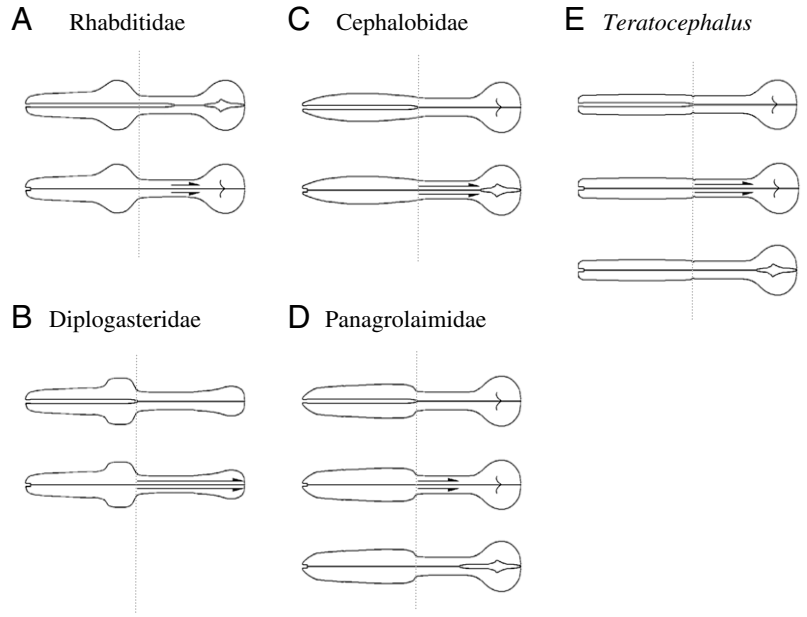
Overall, pharyngeal behaviors differed both with respect to (1) where pumping and peristalsis occurred, and (2) how these motions were coupled, which we term their spatial and coupling patterns, respectively. In the Rhabditidae family, as in *C. elegans*, pumping occurred anteriorly in the corpus and anterior isthmus and posteriorly in the terminal bulb (TB), with peristalsis in the posterior isthmus (Fig. 2A). Further, as in *C. elegans*, corpus, anterior isthmus and TB pumping were all coupled in the Rhabditidae family (Fig. 2A). In the Diplogasteridae family, however, pumping occurred only anteriorly in the corpus with no posterior region of pumping, and peristalsis occurred throughout the isthmus and TB (Fig. 2B). The Diplogasteridae TB also lacks the grinder (Maggenti, 1981; von Lieven and Sudhaus, 2000). Furthermore, in the Diplogasteridae, while the isthmus and TB were coupled for peristalsis, corpus pumping occurred independently (Fig. 2B). In the Cephalobidae family, pumping occurred anteriorly in the corpus and posteriorly in the TB, whereas peristalsis occurred in the entire isthmus (Fig. 2C). While corpus pumping occurred independently, isthmus peristalsis and TB pumping were coupled in the Cephalobidae, such that each isthmus peristalsis always occurred with a TB pump and *vice versa* (Fig. 2C). In the Panagrolaimidae family, pumping occurred anteriorly in the corpus and posteriorly in the posterior isthmus and TB, whereas peristalsis occurred only in the anterior isthmus (Fig. 2D). Furthermore, in the Panagrolaimidae, corpus pumping, anterior isthmus peristalsis, and posterior isthmus/TB pumping all occurred independently of each other (Fig. 2D). In one Panagrolaimidae species, *Panagrellus redivivus*, some posterior isthmus/TB pumps were coupled to corpus pumps, although posterior isthmus/TB pumping also occurred independently. Finally, in the outgroup species *T. lirellus*, pumping occurred anteriorly in the corpus and posteriorly in the TB, and peristalsis occurred in the entire isthmus (Fig. 2E), which is similar to the Cephalobidae family. However, in *T. lirellus*, corpus pumping, isthmus peristalsis, and TB pumping all occurred independently (Fig. 2E), which is more similar to the Panagrolaimidae family. Representative videos of these different patterns in each family are included as supplementary material, and can also be viewed at http://eatworms.swmed.edu/~alan.

By assuming the principle of parsimony, pharyngeal behaviors of the outgroup species, *T. lirellus*, likely represent the ancestral patterns of free-living soil nematodes (both with respect to the spatial and coupling patterns of pumping and peristalsis). Based on this, corpus behaviors remained largely conserved in free-living soil nematodes. As in the ancestral pattern, the corpus pumped in all four families, and pumped independently (i.e. not coupled to isthmus and TB motions) in all families except the Rhabditidae. In contrast, the isthmus and terminal bulb evolved extensive changes from the ancestral

pattern in each family. In the Rhabditidae, the anterior isthmus switched from peristalsis to pumping, and both anterior isthmus and terminal bulb pumping became coupled to the corpus. In the Diplogasteridae family, the TB switched from pumping to peristalsis, and the isthmus and TB became coupled for peristalsis. In the Cephalobidae family, while the spatial pattern of pumping and peristalsis remained ancestral, isthmus peristalsis and TB pumping became coupled. In the Panagrolaimidae family, while the overall coupling pattern remained ancestral, the posterior region of pumping expanded as the posterior isthmus switched from peristalsis to pumping. This model of how pharyngeal behaviors evolved is depicted in Fig. 2F.

Differences in stimulation of isthmus and TB behaviors by the M4 and M2 neurons

Having characterized how pharyngeal behaviors evolved in free-living soil nematodes, we were then interested in how their neuronal regulation may have correspondingly evolved. In *C. elegans*, M4 is required for posterior isthmus peristalsis,



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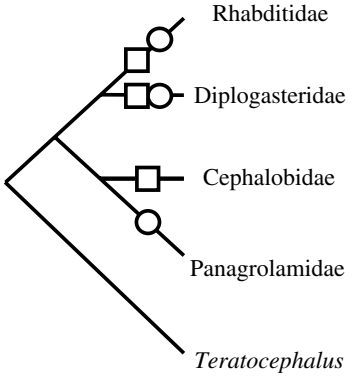

	'Spatial pattern' of isthmus/TB behaviors	'Coupling pattern' of isthmus/TB behaviors
Rhabditidae	**Derived** (PI peristalsis, AI/TB pumping)	**Derived** (AI and TB coupled to corpus)
Diplogasteridae	**Derived** (Isthmus/TB peristalsis)	**Derived** (Isthmus/TB coupled)
Cephalobidae	**Ancestral** (Isthmus peristalsis, TB pumping)	**Derived** (Isthmus/TB coupled)
Panagrolamidae	**Derived** (AI peristalsis, PI/TB pumping)	**Ancestral** (No coupling)
Teratocephalus	**Ancestral** (Isthmus peristalsis, TB pumping)	**Ancestral** (No coupling)

Fig. 2. Evolution of pharyngeal behaviors in free-living soil nematodes. Pharyngeal behaviors in four free-living soil nematode families and an outgroup species (*Teratocephalus lirellus*) are illustrated in A–E, where pumping is represented by open pharyngeal lumens and peristalsis is represented by arrows. The corpus pumped in all four families and pumped independently in all families except the Rhabditidae. The isthmus and terminal bulb (TB), however, exhibited significant differences regarding where pumping/peristalsis occurred, as well as how different parts were coupled in their contractions (see Results for more detailed descriptions). Thin grey lines are drawn between the corpus and isthmus/TB in each schematic to help visualize the largely conserved corpus behaviors and the varied isthmus/TB behaviors. (A) Rhabditidae family (including *C. elegans*): pumping occurred in the corpus, anterior isthmus, and TB, whereas peristalsis occurred in the posterior isthmus. Corpus, anterior isthmus, and TB pumping were coupled. (B) Diplogasteridae family: pumping occurred only in the corpus, whereas peristalsis occurred in the isthmus and TB. Isthmus and TB were coupled to conduct peristalsis. The Diplogasteridae TB lacks the grinder (Maggenti, 1981; Zhang and Baldwin, 1999). (C) Cephalobidae family: pumping occurred in the corpus and terminal bulb, whereas peristalsis occurred in the entire isthmus. Isthmus peristalsis and TB pumping were coupled. (D) Panagrolaimidae: pumping occurred in the corpus, posterior isthmus and TB, whereas peristalsis occurred in the anterior isthmus (AI). Corpus pumping, anterior isthmus peristalsis, and posterior isthmus/TB pumping all occurred independently. (E) *T. lirellus*: pumping occurred in the corpus and TB, whereas peristalsis occurred in the entire isthmus. Corpus pumping, isthmus peristalsis, and TB pumping all occurred independently. (F) A model of how pharyngeal behaviors evolved in the isthmus and terminal bulb of free-living soil nematodes, together with the currently accepted phylogenetic relationships between each family (Blaxter et al., 1998; Felix et al., 2000; Goldstein et al., 1998). Spatial pattern refers to where pumping/peristalsis occurred, whereas coupling pattern refers to how the different motions were coupled. By comparison to the outgroup species, *T. lirellus*, the spatial pattern in the Cephalobidae is ancestral, whereas the coupling pattern in the Panagrolaimidae is ancestral. Open circles and squares on the phylogenetic tree indicate that spatial and coupling patterns, respectively, evolved from an ancestral pattern to a derived pattern in the subsequent lineage. For example, the Rhabditidae lineage is marked with both a circle and a square to denote that both its spatial and coupling patterns are derived. AI, anterior isthmus; PI, posterior isthmus; TB, terminal bulb.

whereas MC and gap junction coupling are important for stimulating corpus pumping and anterior isthmus/TB pumping, respectively (schematic in Fig. 1C). Since corpus pumping occurred independently of isthmus and TB motions in the Diplogasteridae, Cephalobidae and Panagrolaimidae, gap junction coupling to the corpus is unlikely to be important for isthmus and TB excitation in those families. On the other hand, nematode nervous systems are generally well conserved anatomically (Martin et al., 2002), and we were similarly able to identify homologs to most *C. elegans* pharyngeal neurons in the other species (see Fig. 3; identification of ventral corpus neurons was generally less certain, and TB neurons of the Diplogasteridae could not be identified at all). Thus, how might have M4 and MC functions evolved?

Using laser ablations, a technique used in *C. elegans* to inactivate and define the functions of individual neurons (Bargmann and Avery, 1995), we examined M4 functions in select species from the Diplogasteridae, Cephalobidae, and Panagrolaimidae families. In *Pristionchus pacificus* of the Diplogasteridae family, M4 ablation caused a reduction in isthmus/TB peristalsis (Fig. 4A). In *Cephalobus sp.* DWF1301 of the Cephalobidae family, M4 ablation caused a reduction in isthmus peristalsis/TB pumping (Fig. 4B). In *Panagrolaimus sp.* PS1159 of the Panagrolaimidae family, M4 ablation interestingly had no effect on anterior isthmus peristalsis, but caused a specific reduction in posterior isthmus/TB pumping (Fig. 4C). Thus, outside of *C. elegans* and the Rhabditidae family, we found M4 to be an important excitatory neuron as well in the other three free-living soil nematode families. In fact, with the exception of anterior isthmus peristalsis in the Panagrolaimidae, and anterior isthmus/TB pumping in the Rhabditidae, all isthmus/TB behaviors in the four families appeared to respond to M4 stimulation. Yet, M4 varied in its detailed function between each family, both with regards to (1) the exact region of isthmus and TB affected by M4 stimulation, and (2) whether M4 stimulated pumping and/or peristalsis.

Since the source of neuronal regulation for anterior isthmus peristalsis in the Panagrolaimidae family was not revealed by M4 ablations, we conducted laser ablations of additional pharyngeal neurons in *Panagrolaimus sp.* PS1159. Interestingly, we found that ablation of M2, a neuron which has no known function in *C. elegans*, caused a decrease in *Panagrolaimus sp.* PS1159 anterior isthmus peristalsis (Fig. 4C), indicating that M2 was the important excitatory neuron for anterior isthmus peristalsis in the Panagrolaimidae. Further, the Diplogasteridae family had no obvious M2 homolog (see Fig. 3), and in the Cephalobidae family, ablation of M2 caused no phenotypes in *Cephalobus sp.* DWF1301 (data not shown). Thus, M2 appeared to function in the Panagrolaimidae family specifically.

Based on these laser ablation studies, we saw that the isthmus and TB in each family evolved significant differences in their patterns of neuronal stimulation (schematics in Fig. 5). In the Rhabditidae, M4 stimulates posterior isthmus peristalsis, whereas gap junction coupling to the corpus is important for

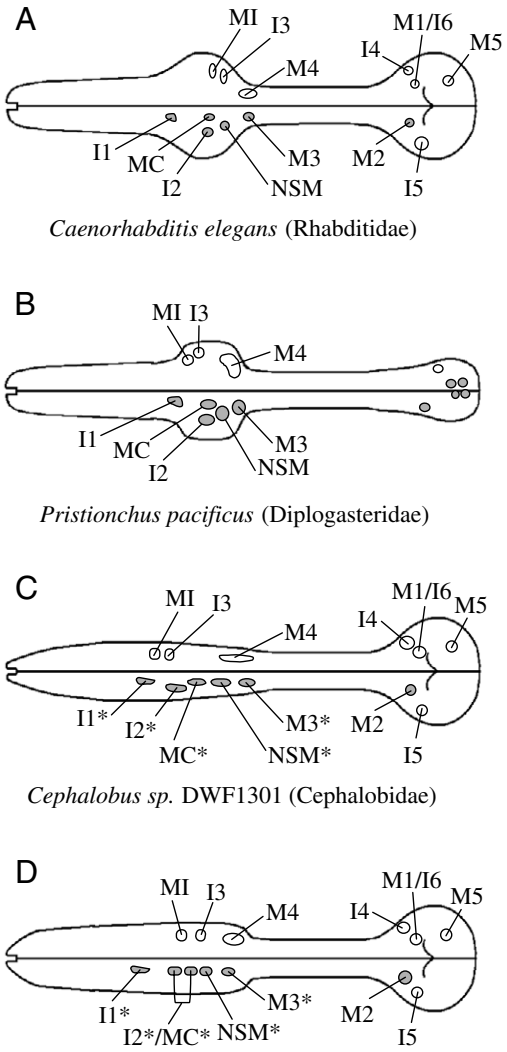

Fig. 3. Identification of *C. elegans* pharyngeal neuron types in other nematodes. (A) The 20 neurons in the *C. elegans* pharynx. There are 14 neuronal types, 8 of which are single neurons (not shaded), and 6 of which are bilaterally symmetric left–right pairs (shaded). Paired neurons are more lateral, whereas single neurons are mostly near the midline, except that M1 is on the right side and I6 and M5 are on the left side. M1 and I6 are drawn as one nucleus because they occupy similar positions in the pharynx, even though they are two distinct neurons (M1 is right, I6 is left). (B–D) Nuclei of putative homologs of *C. elegans* pharyngeal neurons in *Pristionchus pacificus* PS312 (Diplogasteridae), *Cephalobus sp.* DWF1301 (Cephalobidae), and *Panagrolaimus sp.* PS1159 (Panagrolaimidae). Most *C. elegans* pharyngeal neurons were readily identifiable in these other species based on their sizes, shapes and relative positions on DIC microscopy, except for TB neurons in *P. pacificus* (Diplogasteridae), whose relationships to *C. elegans* TB neurons were not apparent. Additionally, assignments of homology in the ventral corpus were often tentative, as indicated by the asterisks, due to differences and inconsistencies in neuronal characteristics compared to *C. elegans*. (Even in *C. elegans*, positions of neurons in the ventral corpus are variable in adults, although they are reproducible in young larvae.)

anterior isthmus/TB pumping; in the Diplogasteridae, M4 stimulates isthmus/TB peristalsis; in the Cephalobidae, M4 stimulates isthmus peristalsis/TB pumping; and in the Panagrolaimidae, M4 stimulates posterior isthmus/TB pumping whereas M2 is important for anterior isthmus peristalsis. Overall, M4 appears to be the central excitatory neuron for isthmus/TB behaviors, whereas M2 and gap junction coupling have specific roles in particular families.

In addition to the changes in isthmus and TB stimulation by the M4 and M2 neurons, we were also interested in evolutionary differences in the function of the MC neurons. Systematic examination of MC function was much more difficult, however, because the homologs of MC and other ventral corpus neurons were harder to identify in non-*C. elegans* nematodes (see above and Fig. 3). Putative MC ablations in *P. pacificus* PS312, a Diplogasteridae species, and *P. redivivus* DA1711, a Panagrolaimidae species (data not shown) suggested that MC may be generally conserved to stimulate corpus pumping, but this hypothesis awaits further future testing.

Killing M4 in *P. pacificus* and M2 in *Panagrolaimus sp.* PS1159 led to moderate decreases in corpus pumping (Fig. 4A,C). While these results might suggest that M4 and M2 contribute to corpus pumping in the Diplogasteridae and Panagrolaimidae families, an alternative explanation is that the effects are actually secondary to the peristalsis defects. In *C. elegans*, accumulation of bacteria in the corpus due to defective peristalsis after M4 ablation can cause reduced corpus pumping (Raizen et al., 1995), and we think a similar effect occurs in M4/M2 ablated *P. pacificus* and *Panagrolaimus sp.* PS1159 animals.

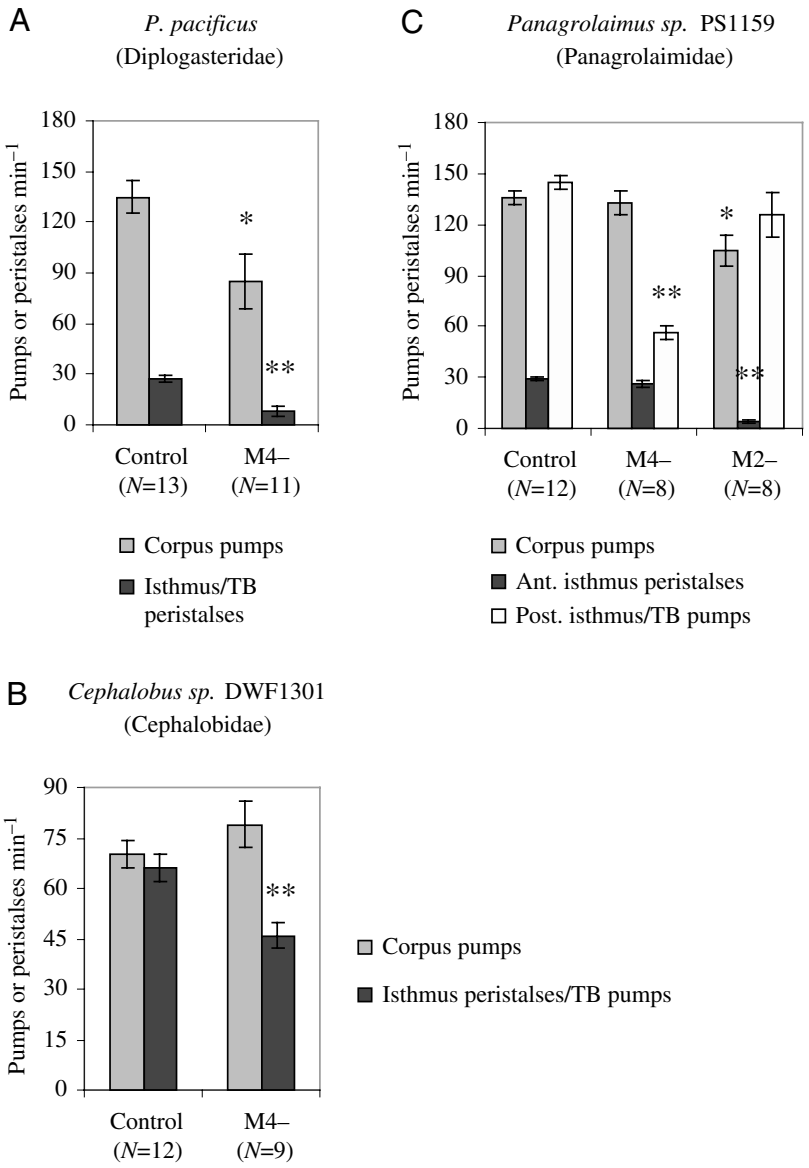

Fig. 4. M4 and M2 stimulated isthmus/terminal bulb behaviors in the Diplogasteridae, Cephalobidae, and Panagrolaimidae families. Laser ablations in select species from the Diplogasteridae, Cephalobidae, and Panagrolaimidae families. (A) *Pristionchus pacificus* PS312 (Diplogasteridae): M4 ablation caused a reduction in isthmus/TB peristalsis and also a decrease in corpus pumping. (B) *Cephalobus sp.* DWF1301 (Cephalobidae): M4 ablation caused a reduction in isthmus peristalsis/TB pumping. (C) *Panagrolaimus sp.* PS1159 (Panagrolaimidae): M4 ablation caused a reduction in posterior isthmus/TB pumping. M2 ablation caused a reduction in anterior isthmus peristalsis and also a reduction of corpus pumping. The effects on corpus pumping of M4 ablation in *P. pacificus* and M2 ablation in *Panagrolaimus sp.* PS1159 are probably secondary to the defects in peristalsis (see Results). Asterisks indicate statistically significant differences from controls by two-tailed *t*-test (**P<0.01, *P<0.05).

Loss of slo-1, a negative regulator of synaptic transmission, activated M4-TB synapses in C. elegans

We wished to determine underlying mechanisms that may be responsible for these differences in pharyngeal behaviors and neuronal functions. As shown above, the exact isthmus/TB regions stimulated by M4 varied in each family. In particular, while M4 stimulation affected terminal bulb behaviors in the Diplogasteridae, Cephalobidae, and Panagrolaimidae families, M4 had no effect on the terminal bulb in the Rhabditidae family. Why might this be? We addressed this question by examining the relationship between M4 and the TB in more detail in the Rhabditidae, using *C. elegans*.

One possible reason could be that M4 does not innervate TB muscles. We thus characterized M4's synaptic pattern in *C. elegans* using *Pceh-28::snb-1::gfp*, a transgene in which the *Pceh-28* promoter drives M4 specific expression (WormBase 2006; http://www.wormbase.org/db/gene/gene?name=ceh-28) of the synaptic vesicle marker SNB-1::GFP (Nonet, 1999). In these transgenic animals, we found punctate clusters of SNB-

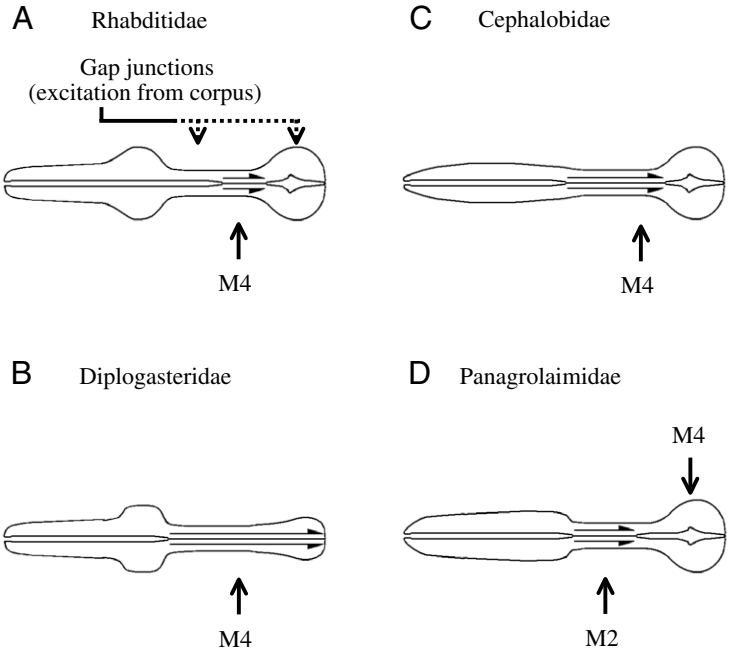

Fig. 5. Schematic of neuronal stimulation in the isthmus and terminal bulb of free-living soil nematodes. The major sources of isthmus and terminal bulb stimulation, as revealed by laser ablations (see Fig. 4 and Results). (A) In the Rhabditidae (*C. elegans*), M4 stimulates posterior isthmus peristalsis, while anterior isthmus/TB pumping is stimulated *via* gap junction coupling to the corpus, as indicated by the broken arrows. (B) In the Diplogasteridae (*P. pacificus*), M4 stimulates isthmus/TB peristalsis. (C) In the Cephalobidae (*Cephalobus sp.* DWF1301), M4 stimulates isthmus peristalsis and TB pumping. (D) In the Panagrolaimidae (*Panagrolaimus sp.* PS1159), M2 stimulates anterior isthmus peristalsis while M4 stimulates posterior isthmus/TB pumping.

1::GFP along M4 axons in both the isthmus and terminal bulb (Fig. 6A), indicating that M4 did in fact innervate the terminal bulb in *C. elegans*. To confirm that the observed punctae truly corresponded to synaptic structures, we also expressed *Pceh-28::snb-1::gfp* in *unc-104* mutants, in which synaptically localized proteins such as SNB-1::GFP should mislocalize to neuronal cell bodies (Nonet, 1999; Nonet et al., 1998) due to synaptic vesicle transport defects (Hall and Hedgecock, 1991; Otsuka et al., 1991). Indeed, in *unc-104* mutants carrying *Pceh-28::snb-1::gfp*, we found SNB-1::GFP mislocalized to the M4 cell body (data not shown).

Therefore, M4 innervates the TB in *C. elegans*. Why then, despite the presence of synapses, is TB behavior unaffected by M4? The gene *slo-1* encodes a Ca^{2+} activated K^+ channel that negatively modulates neurotransmission (Wang et al., 2001), and we observed a suggestive phenotype on EPGs (electropharyngeograms) from *slo-1* mutants.

The EPG is an extracellular current recording of pharyngeal muscles during pumping (Avery et al., 1995; Raizen and Avery, 1994). Peristalsis does not generate EPG currents. MC stimulation of pharyngeal muscles causes small positive spikes on EPGs, which either (1) remain as single positive spikes if MC fails to trigger full muscle action potentials (i.e. they represent MC EPSPs) or (2) are followed by large positive and negative spikes representing full muscle depolarizations and repolarizations, if MC successfully elicits muscle action potentials (Raizen and Avery, 1994; Raizen et al., 1995). In addition to MC activity, activity from the M3 neurons to repolarize pharyngeal muscles during action potentials also appears on EPGs. M3 IPSPs are visible as trains of small negative spikes during the muscle action potential (Raizen and Avery, 1994). MC and M3 are the only neurons whose functions are observed in EPGs (see Fig. 6B), and inactivation of MC and M3 by laser ablations or mutations such as *eat-2* (McKay et al., 2004; Raizen et al., 1995) and *eat-4* (Lee et al., 1999) abolishes their corresponding EPG signals. A schematic of typical EPG signals, including MC and M3 spikes, is shown in Fig. 6B.

Consistent with *slo-1*'s known function, EPGs of *slo-1* mutants included phenotypes indicative of increased MC and M3 neurotransmission, such as larger MC and M3 spikes (Wang et al., 2001; J.-T. A. Chiang and L. Avery, unpublished observations). However, even when MC and M3 functions were removed, *slo-1* EPGs were still different from wild type. Specifically, even though single positive spikes are normally absent from EPGs from MC–M3– wild-type and *eat-2; eat-4* animals (Raizen et al., 1995), we found that single positive spikes were still present in MC–M3– *slo-1* and *eat-2; eat-4; slo-1* animals (Fig. 6D-F). Thus, in a *slo-1* mutant, some neuron other than MC can activate pharyngeal muscles.

We conducted additional laser ablations in *eat-2; eat-4; slo-1* animals to identify the source of these novel single positive spikes. We found that when we ablated M4 in *eat-2; eat-4; slo-1* animals, single positive spikes were largely eliminated (Fig. 6G). Thus, mutating *slo-1* activated the M4 neuron, and the novel single positive spikes in *slo-1* mutants corresponded to M4 EPSPs in pharyngeal muscles. We then wished to test if activation of M4 caused increased pumping as well. Based on M4's innervation pattern, *slo-1* should cause increased pumping in the TB, but not in the corpus. To test this, we examined the effects of *slo-1* in the *eat-5* background. The *eat-5* mutation causes gap junction defects and uncouples the TB from the corpus, which allows TB pumping to be assessed independently of corpus pumping (Starich et al., 1996). Indeed, we found that *eat-5; slo-1* animals had over twofold greater TB pumping than *eat-5* animals (Fig. 6H), consistent with the idea that *slo-1* activated M4 caused increased TB pumping. [We were unable to test if M4 ablation abolished the increased

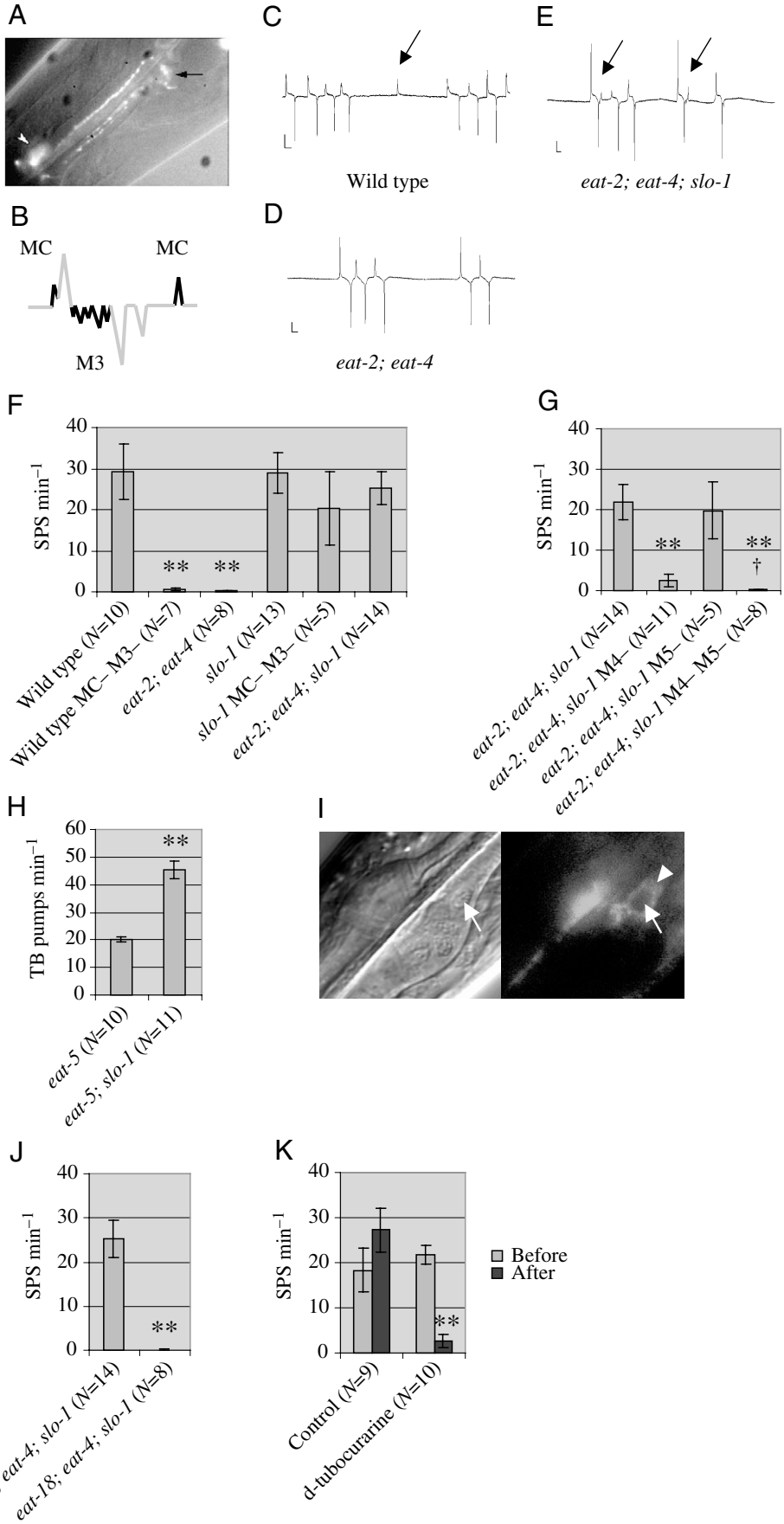

Fig. 6. See next page for legend.

Fig. 6. Loss of *slo-1* in *C. elegans* activated M4-TB synapses. (A) *Pceh-28::snb-1::gfp* transgenic *C. elegans* animals showed punctate expression of SNB-1::GFP, a synaptic vesicle marker, along M4 axons in the isthmus and terminal bulb. White arrowhead points to the M4 cell body. Black arrow points to punctae in the TB. (B) A schematic of *C. elegans* EPGs (electropharyngeograms), a current recording from pharyngeal muscles during pumping, where positive and negative spikes correspond to muscle depolarizations and repolarizations, respectively. Neuronal stimulation by MC causes small positive spikes, which either remain as single positive spikes, i.e. MC EPSPs, if the MC stimulation did not induce muscle action potentials, or are followed by large positive and negative spikes if full muscle action potential was triggered. Inhibition by the M3 neurons sometimes occurs, which contributes to pharyngeal muscle repolarization during action potentials, and is seen as small trains of negative spikes on EPGs. Illustrated in the schematic are one full action potential on the left, and one single positive spike (resulting from an MC EPSP) on the right. The neurogenic spikes (i.e. due to MC and M3) are drawn in black, whereas myogenic spikes are drawn in gray. If MC and M3 are inactivated, then the resulting EPGs contain only myogenic spikes. (C) An example of a single positive spike due to an MC EPSP in wild-type *C. elegans* EPG, indicated by the black arrow. (D) *eat-2; eat-4* EPGs lacked MC and M3 spikes, including single positive spikes (*eat-2* and *eat-4* remove MC and M3 functions, respectively). (E) *eat-2; eat-4; slo-1* EPGs contained single positive spikes, indicated by the black arrows. (F) Quantification of single positive spikes (SPS) in wild-type and *slo-1* mutant backgrounds, with and without MC/M3 function, either by laser ablations or the *eat-2* and *eat-4* mutations. (G) Laser ablation of M4 drastically reduced single positive spikes (SPS) in *eat-2; eat-4; slo-1* animals, whereas ablation of another *C. elegans* neuron, M5, did not. Ablation of M4 and M5 entirely eliminated single positive spikes, suggesting slight activity by M5 as well. (H) The *eat-5* mutation allows the TB to pump independently of the corpus (Starich et al., 1996), and *slo-1* increased TB pumping in the *eat-5* background. (I) Perinuclear expression of *Pslo-1::SLO-1::GFP* in the M4 neuron. The white arrows point to the M4 nucleus. The white arrowhead points to GFP signal surrounding the M4 nucleus. (J) *eat-2* encodes a nicotinic channel subunit specific to the MC-corpus neuromuscular junction, whereas *eat-18* is required for the surface expression of all pharyngeal nicotinic channels, including in the terminal bulb. *eat-2; eat-4; slo-1* animals had single positive spikes, but *eat-18; eat-4; slo-1* animals did not. (K) EPGs of *eat-2; eat-4; slo-1* animals were recorded in standard conditions, followed by subsequent addition of d-tubocurarine, a nicotinic channel antagonist, to 100 μmol l^{-1}, or control saline (see Materials and methods for details). 100 μmol l^{-1} d-tubocurarine decreased single positive spikes in *eat-2; eat-4; slo-1* animals (compared to before treatment), whereas control treatment did not (compared to before treatment). Scale bars in C–E indicate 100 pA and 100 ms. Asterisks indicate statistically significant differences from controls by two-tailed *t*-test (***P*<0.01); see Materials and methods for the statistical analysis performed in Fig. 6K. Dagger in G indicates statistical significance compared to M4 ablation by two-tailed *U*-test (†*P*<0.05).

TB pumping in *eat-5; slo-1* animals, because M4 ablation causes starvation (Avery and Horvitz, 1987) with confounding effects of increased and irregular pumping (Avery and Horvitz, 1990; Raizen et al., 1995).] Finally, if *slo-1* acted directly in the M4 neuron, M4 should also express *slo-1*. To determine the expression pattern of *slo-1*, we examined the expression of *Pslo-1::SLO-1::GFP*, a GFP fusion transgene containing a 5.2 kb *Pslo-1* promoter. Indeed, expression was seen in M4 (Fig. 6I), consistent with *slo-1* acting directly in the M4 neuron. Expression was also detected in most other pharyngeal neurons (data not shown), consistent with *slo-1*'s aforementioned effects on other pharyngeal neurons, such as MC and M3.

Together, the novel M4 dependent single positive spikes and increased TB pumping suggested that loss of *slo-1* activated M4 to stimulate TB pumping. Since *slo-1* encodes a negative modulator of neurotransmission (Wang et al., 2001), these results suggested that *C. elegans* M4–TB synapses can function, but their synaptic activity is normally reduced in wild-type *C. elegans* to a level where M4 has no effect on TB behavior, i.e. M4–TB synapses are silenced in *C. elegans*. Based on this, we suggest that silencing of M4–TB synapses may account for the evolutionary differences in M4–TB stimulation between the Rhabditidae and other free-living soil nematode families.

Another gene that is required for MC to stimulate pharyngeal pumping is *eat-18* (McKay et al., 2004; Raizen et al., 1995). However, whereas *eat-2* encodes a nicotinic channel subunit specifically localized to the MC-corpus neuromuscular junction, *eat-18* is a gene that is required for the surface expression of nicotinic receptors throughout the pharynx (McKay et al., 2004). Interestingly, in contrast to *eat-2; eat-4; slo-1* animals, we found that *eat-18; eat-4; slo-1* animals lacked single positive spikes (Fig. 6J). We then also tested whether the nicotinic antagonist d-tubocurarine (Raizen et al., 1995) can affect single positive spikes, and indeed, 100 μmol l^{-1} d-tubocurarine drastically reduced the number of single positive spikes in *eat-2; eat-4; slo-1* EPGs (Fig. 6K). Together, the *eat-18* and d-tubocurarine experiments indicated that M4-TB synapses are cholinergic, and stimulate the TB muscles *via* nicotinic receptors.

Isthmus/TB pumping/peristalsis rates respond to food density changes

With regard to pumping and peristalsis patterns of free-living soil nematode families, we described above that corpus pumping generally occurred independently of isthmus and TB motions (Fig. 2A–D) (except coupled corpus/anterior isthmus/TB pumping in the Rhabditidae). We found that when we diluted the food in the animals' surroundings (see Materials and methods for experimental setup), corpus pumping rates were increased or similar to those in normal food conditions, but isthmus/TB pumping/peristalsis rates were generally reduced (Fig. 7). This suggested that independent regulation of corpus and isthmus/TB contraction rates may be useful for responding to changes in food density. When food is scarce (i.e. the dilute condition), the corpus still pumps rapidly to maximize the amount of food ingested; yet, since less food is ingested overall, isthmus and TB contraction rates are down-regulated for energy efficiency as less food needs to be passed posteriorly and chewed by the grinder. Additionally, since

reducing food density has effects similar to ablating M4 (compare the effects of M4 ablation in Fig. 4 with decreasing food density in Fig. 7), we hypothesize that M4 may be important for mediating the food density response of isthmus/TB contractions.

Discussion

To explore the utility of *C. elegans* and related nematodes for studying behavioral evolution, we examined pharyngeal behaviors and neuronal functions in free-living soil nematodes. Overall, pharyngeal behaviors in each major family consisted of different patterns of pumping and peristalsis. Based on parsimony, the outgroup *T. lirellus* likely represents the ancestral pattern, with corpus pumping, isthmus peristalsis, and TB pumping, each of which occurring independently. In the Rhabditidae, the anterior isthmus switched to pumping and anterior isthmus/TB pumping became coupled to corpus pumping; in the Diplogasteridae, the TB switched to peristalsis and functioned together with the isthmus, possibly coincident with an anatomical reduction of TB structures; in the Cephalobidae, isthmus peristalsis became coupled with TB pumping; and in the Panagrolaimidae, the posterior isthmus switched to pumping. We also found differences in neuronal function. M4 was an important isthmus/TB excitatory neuron in each family, and in all cases M4 function can be understood as carrying information about food density to the isthmus/TB. However, M4's exact downstream function varied from family to family between: stimulation of posterior isthmus peristalsis (Rhabditidae), isthmus/TB peristalsis (Diplogasteridae), isthmus peristalsis/TB pumping (Cephalobidae), and posterior isthmus/TB pumping (Panagrolaimidae). In addition, M2 functioned specifically in the Panagrolaimidae to stimulate anterior isthmus peristalsis, and gap junction coupling to the corpus appeared to be specifically important for anterior isthmus/TB pumping in the Rhabditidae.

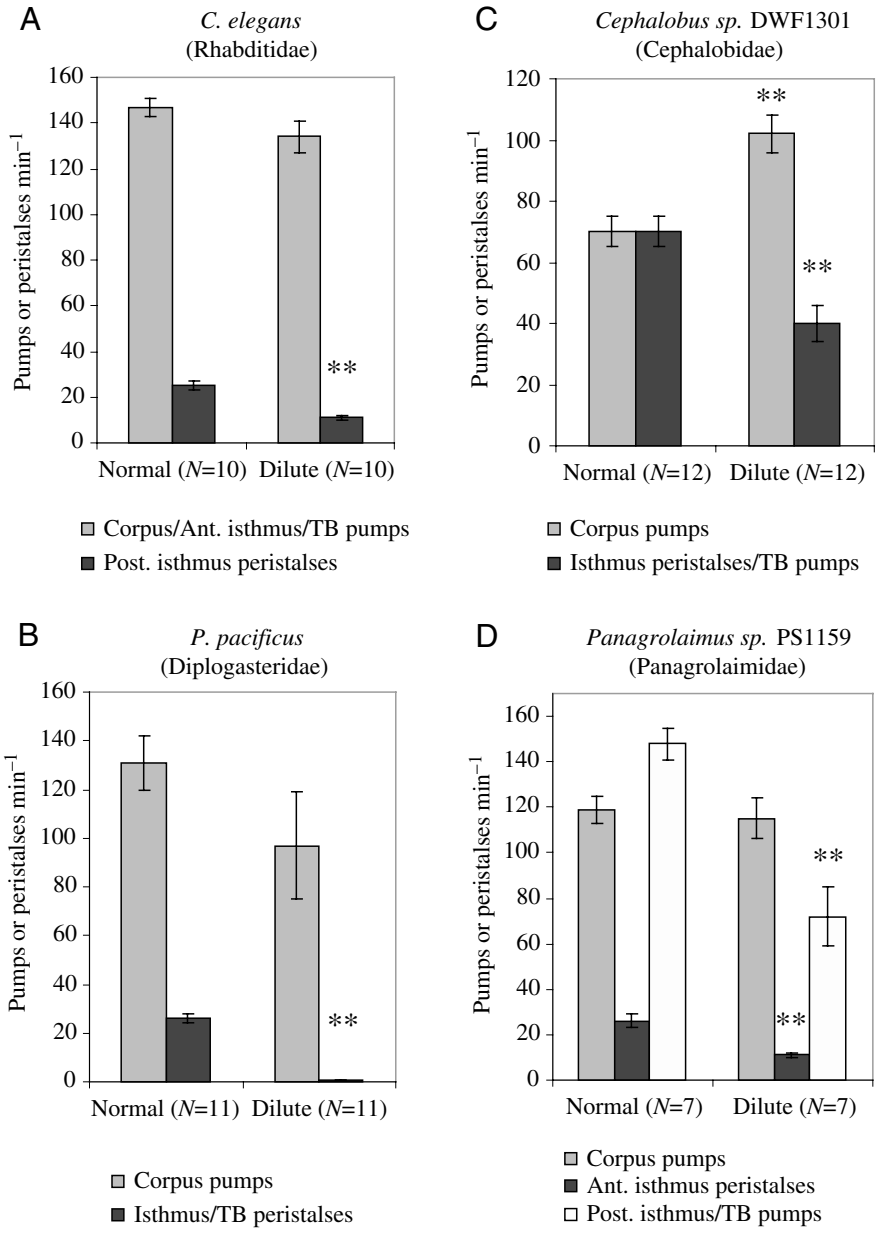

Fig. 7. Food density dependence of isthmus/TB pumping and peristalsis. When (A) *C. elegans*, (B) *P. pacificus*, (C) *Cephalobus sp.* DWF1301, or (D) *Panagrolaimus sp.* PS1159 animals were placed in dilute food conditions compared to normal conditions (see Materials and methods), isthmus/TB pumping/peristalsis rates were reduced whereas corpus pumping rates were unchanged or increased. Asterisks indicate statistically significant differences between dilute and normal conditions by two-tailed *t*-test (**P<0.01).

Altogether, these observations form a broad overview of how pharyngeal behaviors and neuronal stimulation evolved in free-living soil nematodes, and provide a framework for investigating the underlying cellular and genetic mechanisms of change. Some details, however, will require future clarification. With respect to pharyngeal behaviors, for example, it is unclear which evolved first in the Rhabditidae family: (1) switch of anterior isthmus from peristalsis to pumping, or (2) coupling of anterior isthmus/TB pumping to the corpus. In *Poikilolaimus regenfussi*, a basal Rhabditidae species (Kiontke and Fitch, 2005), we observed anterior isthmus pumping, but TB pumps were often weakly coupled to the corpus, suggesting incomplete gap junction coupling. This observation is potentially informative, but

since the phylogenetic relationships between *P. regenfussi* and other Rhabditidae species are still under investigation (Kiontke and Fitch, 2005), it is unclear whether *P. regenfussi* signifies that gap junction coupling evolved after anterior isthmus pumping, or if gap junctions were secondarily weakened in the *P. regenfussi* lineage. This can be resolved with future clarification of the Rhabditidae phylogeny.

With respect to how neuronal stimulation of the pharynx evolved, the major gap in our understanding is the ancestral functions of the M4 and M2 neurons. We attempted laser ablations in *T. lirellus*, but were unsuccessful due to technical problems (see Materials and methods). Further, other species that are closely related to the free-living soil nematodes, such as the Plectidae (Blaxter et al., 1998), do not have peristalsis and do not have three-part pharynxes (von Lieven, 2003; J.-T. A. Chiang, unpublished), and were therefore not useful as outgroups. Thus, determination of the ancestral functions of pharyngeal neurons await the availability of more favorable outgroup species.

Having characterized how pharyngeal behaviors and their neuronal stimulation evolved, we then focused on understanding the mechanisms by which M4–TB stimulations are absent specifically in the Rhabditidae family. In *C. elegans*, we found that M4 actually innervates the TB, even though it has no detectable function there in wild type. Further, *slo-1* encodes a BK type Ca^{2+}-activated K^+ channel that negatively modulates neurotransmission (Wang et al., 2001), and novel EPG spikes and increased TB pumping suggested that M4–TB stimulations occurred in *slo-1* mutants. Thus, M4–TB synapses in *C. elegans* apparently have the capability for function, but neurotransmission at these synapses is reduced to undetectable or irrelevant levels. Based on this, we propose that M4–TB synapses have been silenced during the evolution of the Rhabditidae family, leading to the lack of M4–TB stimulations. Interestingly, using the *eat-18* mutation and the drug d-tubocurarine, we also demonstrated the cholinergic nature of M4–TB synapses. TB muscles in *C. elegans* had been shown to express surface nicotinic channels (McKay et al., 2004), but their functional significance was unclear. An admittedly speculative, but potentially interesting, possibility is that these channels represent nonfunctional remnants from previously active M4–TB synapses.

Although the *slo-1* phenotypes suggest that modulation of neurotransmission at M4–TB synapses may be important for the absence of M4–TB stimulation in the Rhabditidae, we emphasize that there are clearly alternative possibilities – for example, changes in the number of M4–TB synapses, or changes in the way TB muscles respond to M4 stimulation. Additional future studies, including characterization of M4–TB synapses in the non-Rhabditidae families, will help distinguish between the different possibilities. Nonetheless, we have also identified several additional *C. elegans* genes that can activate M4–TB synapses when mutated, and they also encode presynaptic ion channel subunits that modulate neurotransmission, such as *unc-2* and *unc-36*, which encode subunits of a voltage-gated Ca^{2+} channel (M. Steciuk, unpublished observations). Thus, modulation of neurotransmission is an attractive hypothesis, and we hope to identify evolutionary differences in these genes that correlate with the activity/inactivity of M4–TB synapses in each family. In particular, differential expression of alternatively spliced BK type Ca^{2+}-activated K^+ channels are known to be of physiological importance (i.e. Atkinson et al., 2000; Rosenblatt et al., 1997), and an interesting hypothesis is that differential expression of a specific SLO-1 isoform in M4, in the Rhabditidae family, is critical for inactivating M4–TB synapses.

Given the observed evolutionary differences in pharyngeal behaviors, what functional implications might they have? That is, are they advantageous or adaptive in any way? As shown above (Fig. 7), the general lack of corpus and isthmus/TB coupling appeared to be useful for responding to changes in food density. Further, in the Rhabditidae family, the switch in the anterior isthmus from peristalsis to pumping was likely important, as computational modeling shows that anterior isthmus pumping drastically enhances the efficiency of food transport by the corpus (Avery and Shtonda, 2003).

On the other hand, other evolutionary differences in pharyngeal behaviors did not have obvious functional consequences. In several cases, they even appeared paradoxical. For example, why TB pumping became coupled to corpus pumping in the Rhabditidae family is somewhat perplexing, since this prevented the ability to independently regulate TB pumping rates in response to food density changes. In the Diplogasteridae, the loss of the TB grinder and TB pumping is also perplexing, since the ability to chew was lost. Other researchers have also been intrigued by this (von Lieven and Sudhaus, 2000; Zhang and Baldwin, 1999), and while the reason is not obvious, it may be related to the diversified diet of the Diplogasteridae – it is the only free-living soil nematode family known to feed on non-bacterial food sources (von Lieven and Sudhaus, 2000).

Our results demonstrate the utility of comparative studies between *C. elegans* and other nematodes for studying behavioral evolution. One major advantage of studying *C. elegans* is its relative simplicity, a feature shared by nematodes in general. Thus, as in our study of the pharynx, the evolution of nematode behaviors and neuronal functions can be characterized in terms of very precise and discrete changes. In particular, the use of laser ablations in comparative studies may be a powerful way to examine how neuronal functions evolve at the level of single neurons and small neuronal circuits. To complement these advantages, the wealth of techniques and resources in *C. elegans* can be helpful for understanding the underlying cellular and genetic mechanisms of change, as with our study on M4–TB stimulation. Currently, molecular and genetic techniques are being actively developed for non-*C. elegans* nematodes as well, such as in *Pristionchus pacificus* (Kenning et al., 2004; Srinivasan et al., 2003; Srinivasan et al., 2002), which will be of value. For similar reasons to these, there has already been much interest in using *C. elegans* and related nematodes as models for studying the evolution of

developmental mechanisms (i.e. Dichtel et al., 2001; Felix et al., 2000; Sommer, 1997), and we think that comparative studies in *C. elegans* and related nematodes can also provide useful models for investigating the mechanisms that underlie behavioral and neuronal evolution.

List of abbreviations

DIC	differential interference contrast
EPG	electropharyngeogram
5HT	serotonin
PCR	polymerase chain reaction
TB	terminal bulb
dTC	d-tubocurarine

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