EVOLUTION OF PHARYNGEAL FEEDING BEHAVIORS
IN FREE-LIVING SOIL NEMATODES

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DEDICATION

First and foremost, I would like to thank my mentor, Leon Avery, for his guidance throughout my research. Leon is both a superb scientist and a wonderful teacher, and he creates an amazing laboratory environment for fostering the student's intellectual and personal growth. I also want to extend my deepest thanks to all Avery lab members – Jim McKay, Boris Shtonda, Mark Steciuk, Sarah Stroud-Anselmo, Youngjai You, as well as other UTSW students, in particular, Jeff Cheng, Yuichi Nishi, Eric Rogers, Jaemyoung Suh, Rhea Sumpter, and Elie Traer for their friendship and support during these years. My thesis committee, Ege Kavalali, Thomas Sudhof, and James Waddle, provided many terrific suggestions in guiding my research, and everyone in the MSTP and the Department of Molecular Biology, especially Robin Downing, Dennis McKearin, Rod Ulane, Sue Lott and Stephen Johnson, gave me wonderful assistance in all things big and small. Finally, I would like to thank mom, dad, Anna, and Devi for their love and support during these important years of my life.

EVOLUTION OF PHARYNGEAL FEEDING BEHAVIORS IN FREE-LIVING SOIL NEMATODES

by

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To explore using nematodes as a model for studying behavioral evolution, I examined pharyngeal behaviors in free-living soil nematodes related to *Caenorhabditis elegans*.

The nematode pharynx is divided into three regions: corpus, isthmus, and terminal bulb. Pharyngeal behaviors consist of stereotyped patterns of two motions: pumping and peristalses. I observed pharyngeal behaviors in multiple species, and constructed the following evolutionary model. In the ancestor of free-living soil nematodes, the pharynx had corpus pumping, isthmus peristalses, and terminal bulb pumping, each of which occurred independently. In the Rhabditidae family, the anterior isthmus switched to pumping, and anterior isthmus and terminal bulb pumping became coupled to corpus pumping. In the Diplogasteridae family, the terminal bulb switched to peristalses. In the Cephalobidae family, isthmus peristalses and terminal bulb pumping became coupled. And in the Panagrolaimidae family, the posterior isthmus switched to pumping.

The above changes in isthmus and TB behaviors suggested corresponding changes in their neuronal regulation. Using laser ablations, I found that M4's function evolved significantly: M4 stimulated posterior isthmus peristalsis (Rhabditidae), isthmus/terminal bulb peristalsis (Diplogasteridae), isthmus peristalsis and terminal bulb pumping (Cephalobidae), and posterior isthmus/terminal bulb pumping (Panagrolaimidae). Yet,

increased food activated M4 activity in all families. Thus, M4 appeared to be a general "food sensor" neuron, which was co-opted during evolution to perform different downstream functions. Additionally, M2 stimulated anterior isthmus peristalsis in the Panagrolaimidae.

Using *Caenorhabditis elegans*, I investigated possible molecular/genetic causes for the above changes. Why is the terminal bulb unaffected by M4 in *Caenorhabditis elegans* and the Rhabditidae? I found that mutating *slo-1* activated M4-terminal bulb stimulations, suggesting that alterations in synaptic transmission silenced M4-terminal bulb synapses. Also, why does M2 stimulate peristalses in the Panagrolaimidae, and M4 in the other families? I found *ceh-28* important for M4 to stimulate peristalsis in *Caenorhabditis elegans*, but M2 also had the potential for *ceh-28* expression. This suggested a genetic/molecular link between the two neurons, and *ceh-28* related mechanisms may determine which neuron stimulates peristalsis.

Overall, I characterized how pharyngeal behaviors evolved at the behavioral, neuronal, and genetic levels. These results suggested the utility of nematodes for studying behavioral evolution.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

5HT – serotonin

AI – anterior isthmus

DIC – differential interference contrast (microscopy)

dTC – d-tubocurarine

EPG – electropharyngeogram

GFP – green fluorescent protein

L1 – first larval stage in development

L2 – second larval stage in development

L3 – third larval stage in development

PCR – polymerase chain reaction

PI – posterior isthmus

TB – terminal bulb

CHAPTER ONE

General Introduction

The use of *C. elegans* and related nematodes for studying the evolution of behaviors

The model organism *Caenorhabditis elegans* is an anatomically simple organism that displays reproducible and easily observed behaviors, such as feeding, mating, defecation, and chemotaxis (Riddle, Blumenthal et al. 1997). The neuronal and molecular control of *C. elegans* behaviors can be analyzed to a great level of detail using various techniques. For example, laser ablations (Bargmann and Avery 1995) can define the functions of single neurons and small neuronal circuits, and various genetic techniques such as RNAi (Kamath and Ahringer 2003) and transgenics (Mello, Kramer et al. 1991) can elucidate the molecular mechanisms that underlie *C. elegans* behaviors.

Recently, our laboratory has become interested in expanding the studies in *C. elegans* to other nematode species, to explore the use of nematodes as a general model for studying how behaviors evolve. Using the well-studied *C. elegans* behaviors as starting points, we wished to understand what differences may have evolved in other nematode species, and also what neuronal and molecular changes may be important for the behavioral differences. While other comparative studies of nematode behaviors have been previously conducted, they usually focused more on evolutionary similarities, with the goal of demonstrating how knowledge from *C. elegans* can be used to understand other nematodes, and vice versa. For example, studies in the parasite *Ascaris* focused on the anatomical and functional similarities to *C. elegans* in the ventral cord neurons used for locomotion (Stretton, Fishpool et al. 1978; Johnson and Stretton 1980; Walrond, Kass et al. 1985), and studies in the parasite *Strongyloides* focused on the similarities to *C. elegans* in amphid sensory neurons and their control of chemotaxis (Ashton, Li et al. 1999; Forbes, Ashton et al. 2004). In contrast, our

goal was to focus explicitly on the evolutionary differences in nematode behaviors, and the neuronal and molecular mechanisms by which these differences arose.

The model – pharyngeal behaviors in free-living soil nematodes

Nematodes feed using a neuromuscular organ situated near the head, termed the pharynx. The feeding behaviors of the *C. elegans* pharynx consist of two muscle motions, "pumping" and "peristalses", which occur in well-defined, stereotyped patterns, and are regulated by a small, self-contained pharyngeal nervous system. Overall, the neuronal and molecular basis of pharyngeal feeding behaviors has been well characterized in *C. elegans* (Avery and Thomas 1997). In contrast, feeding behaviors in other nematode species are not as well understood. In particular, *C. elegans* is a free-living soil nematode (nematodes can be classified by whether they are free-living or parasitic, and soil-dwelling or aquatic), and studies in the pharyngeal behaviors of other free-living soil nematodes have been rather limited (Doncaster 1962; Mapes 1965; von Lieven 2003). Additionally, those previous studies were typically restricted to macroscopic observations of pharyngeal behaviors, but did not address any underlying neuronal and genetic mechanisms.

Thus, for this dissertation project, my goal was to study how feeding evolved in free-living soil nematodes – at the behavioral, as well as the neuronal and molecular levels. I began with direct observations of pharyngeal behaviors in a broad range of species, to obtain a systematic model of how these behaviors evolved in free-living soil nematodes. Then, to understand what changes in neuronal function contributed to the behavioral differences, I used laser ablations to define the functions of homologous pharyngeal neurons. And finally,

I analyzed genes in *C. elegans* whose mutant phenotypes or expression patterns suggested possible genetic/molecular mechanisms by which the behavioral and neuronal differences evolved. Overall, this study provides a basic framework for understanding pharyngeal behaviors in free-living soil nematodes, and demonstrates the utility of nematodes as a system for studying how behaviors evolve.

CHAPTER TWO

Evolution of pharyngeal behaviors

in free-living soil nematodes

Introduction

Background – Pharyngeal feeding behaviors 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). Two types of motions, "pumps", defined as single, distinct contractions that open the pharyngeal lumen, and "peristalses", defined as posteriorly sweeping contraction waves, occur in well-defined, stereotyped patterns (Avery and Horvitz 1987; Avery and Horvitz 1989; Avery and Thomas 1997). Anteriorly, pumping occurs in the corpus and the anterior isthmus (AI) to suck in bacteria, and posteriorly, pumping occurs in the terminal bulb (TB) to break up ingested bacteria with the grinder (Fig. 1B). Peristalsis occurs in between the two regions of pumping, i.e. in the posterior isthmus (PI), and transports food from the anterior isthmus to the TB (Fig. 1B). Pumping in the corpus, AI, and TB are all coupled, i.e. they all contract together with each *C. elegans* pump (Fig. 1B), whereas PI peristalsis occurs separately from pumping. Coupled pumping occurs due to gap junctions between the corpus and the isthmus (Starich, Lee et al. 1996), such that whenever the corpus is stimulated to pump by the MC pharyngeal neurons (Raizen, Lee et al. 1995; McKay, Raizen et al. 2004), the excitations spread rapidly to the AI and TB, causing them to pump simultaneously (Starich, Lee et al. 1996 and Mark Steciuk, personal communication).

Results

The isthmus and terminal bulb evolved differences regarding where pumping and peristalses occurred, and how pumping and peristalses are coupled

Outside of *C. elegans*, observations of pharyngeal feeding behaviors in free-living soil nematodes have been rather limited (Doncaster 1962; Mapes 1965; von Lieven 2003). Thus, I observed the pharyngeal motions in a broad selection of species from each of the four major free-living soil nematode families (Chitwood and Chitwood 1974; Blaxter, De Ley et al. 1998) – the Rhabditidae (which includes *C. elegans*), the Diplogasteridae, the Cephalobidae, and the Panagrolaimidae (see Materials and Methods for strain names). In addition, to help determine the ancestral pattern of pharyngeal behaviors and the history of evolutionary changes, I also observed an outgroup species, *Teratocephalus lirellus*, a close relative to the free-living soil nematodes (Blaxter, De Ley et al. 1998). Anatomically, the pharynxes in all these species are divided into the corpus, isthmus, and terminal bulb (albeit with minor anatomical variations -- see Chitwood and Chitwood 1974; Maggenti 1981; Zhang and Baldwin 1999; von Lieven and Sudhaus 2000; von Lieven 2003). Yet behaviorally, each of the four families, and also *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 different behaviors were coupled. In the Rhabditidae family (Fig. 2A), as in *C. elegans*, pumping occurred anteriorly in the corpus and anterior isthmus (AI) and posteriorly in the terminal bulb (TB), with peristalses in between in the posterior isthmus (PI). Further, as in *C. elegans*, corpus, AI, and TB pumping were all coupled in the Rhabditidae family (Fig. 2A). In the Diplogasteridae family (Fig. 2B), however, pumping occurred only anteriorly in the corpus with no posterior region of pumping, and peristalsis occurred throughout the isthmus and terminal bulb (note that the terminal bulb also lacked

the grinder). And, in the Diplogasteridae (Fig. 2B), corpus pumping and isthmus/TB peristalses occurred independently of each other. In the Cephalobidae family (Fig. 2C), pumping occurred anteriorly in the corpus and posteriorly in the TB, whereas peristalses occurred in between in the entire isthmus. Further, while corpus pumping occurred independently, isthmus peristalsis and TB pumping were coupled in the Cephalobidae (Fig. 2C), such that each isthmus peristalsis was always accompanied by a TB pump. In the Panagrolaimidae family (Fig. 2D), pumping occurred anteriorly in the corpus and posteriorly in both the posterior isthmus (PI) and terminal bulb (TB), whereas peristalses occurred in the anterior isthmus (AI). And, in the Panagrolaimidae (Fig. 2D), corpus pumping, AI peristalses, and PI/TB pumping all occurred independently of each other (in one species, *Panagrellus redivivus*, some PI/TB pumps were coupled to corpus pumps, although PI/TB pumping also occurred independently). And finally, in the outgroup species *T. lirellus* (Fig. 2E), pumping occurred anteriorly in the corpus and posteriorly in the TB, and peristalsis occurred in the entire isthmus, which is similar to the Cephalobidae family. However, in *T. lirellus* (Fig. 2E), corpus pumping, isthmus peristalsis, and TB pumping all occurred independently, which is more similar to the Panagrolaimidae family. Representative videos of these different pharyngeal behaviors are listed in Appendix A.

Using the principle of parsimony, where one assumes the most likely evolutionary history to be the one which requires the fewest evolutionary changes, I deduced that the pharyngeal behaviors of *T. lirellus* represented the ancestral pattern in free-living soil nematodes. Comparing the behaviors of each family with this ancestral pattern, it then appeared that corpus behaviors remained largely conserved in the evolution of free-living soil

nematodes. As in *T. lirellus*, the corpus pumped in all four families. Further, as in *T. lirellus*, the corpus also pumped independently (i.e. not coupled to isthmus and TB motions) in all families except the Rhabditidae.

In contrast, in each of the four families isthmus and terminal bulb behaviors evolved significantly from the ancestral pattern. Compared to the ancestral pattern, the anterior isthmus in the Rhabditidae family switched to pumping, whereas peristalsis became restricted to the posterior isthmus, 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 thus peristalsis occurred throughout the isthmus and TB. In the Cephalobidae family, although the positions of peristalsis and pumping did not change, isthmus peristalses and TB pumping became coupled. And in the Panagrolaimidae family, the posterior isthmus switched to pumping, and the region of peristalsis became restricted to the anterior isthmus.

This model of how pharyngeal behaviors evolved in free-living soil nematodes is depicted in Fig. 3, together with the presently accepted phylogenetic relationships between the families (Blaxter, De Ley et al. 1998; Goldstein, Frisse et al. 1998; Felix, De Ley et al. 2000). Altogether, isthmus and TB behaviors in the Cephalobidae and Panagrolaimidae families contained a mixture of ancestral and derived characteristics, whereas those of the Diplogasteridae and Rhabditidae appeared to be entirely derived.

Food density dependence of isthmus/TB contractions

As described, I found that corpus pumping generally occurred independently of isthmus and TB motions in free-living soil nematodes (coupling of corpus pumping with AI

and TB pumping in *C. elegans* and other Rhabditidae species was the exception). Why might this be? One functional consequence is that the rates of corpus pumping and isthmus/TB contractions can be regulated independently, which appeared to be a useful feature, based on the experiment below.

When I diluted the food (i.e. bacteria) surrounding the animals (see Materials and Methods for experimental setup), I found that isthmus/TB pumping/peristalsis rates were generally reduced in species from each of the four families (Fig. 4A-D). In contrast, corpus pumping rates were either similar or increased (Fig. 4A-D). Intuitively, this made sense: by maintaining frequent corpus pumping, the animals could still ingest as much food as possible, but by lowering isthmus and TB contractions, the animals could be more efficient in processing the decreased amounts of food sucked in by the corpus. Thus, the lack of coupling between the corpus and the isthmus/TB appeared to be a useful design feature, at least for responding to changes in food density.

Discussion

The model of how pharyngeal feeding behaviors evolved in free-living soil nematodes

Based on observations in four major families and one outgroup species, I derived a model of how pharyngeal behaviors evolved in free-living soil nematodes (Fig. 3). While the model provided a relatively complete overview, certain aspects could be resolved in greater detail. For example, which evolved first in the Rhabditidae family: coupling to the corpus, or anterior isthmus pumping? Currently, the detailed phylogenetic relationships within the Rhabditidae and other families are still being clarified (Felix, De Ley et al. 2000). Once they

are resolved, then observations in additional species (selected based on useful phylogenetic positioning) might further improve the model of how pharyngeal behaviors evolved.

Additionally, since I examined only one outgroup species, *T. lirellus*, the inferred ancestral pattern of pharyngeal behaviors should be taken with some caution. Information from additional outgroup species would have been useful. Unfortunately, other common species that are closely related to free-living soil nematodes, such as the Plectidae family (Blaxter, De Ley et al. 1998), do not have peristalses and do not have three-part pharynxes (von Lieven 2003 and my unpublished observations), and thus did not provide useful comparisons.

Finally, since I focused exclusively on free-living soil nematodes, it may also be of interest to compare pharyngeal feeding behaviors in parasitic and/or aquatic nematodes. In cases where their pharyngeal anatomies and feeding behaviors have been examined (Mapes 1965; Mapes 1965; Mapes 1966; Lee 2002), the results suggest dramatic differences compared to *C. elegans* and free-living soil nematodes. Thus, while parasitic and aquatic species may show interesting differences, the magnitude and complexity of those differences may also preclude straightforward analyses of the exact evolutionary changes that occurred.

Functional implications of the evolutionary differences in pharyngeal behaviors

Given the variety of feeding behaviors that I observed, a natural question to ask is: what functional implications might they have? I.e., are they advantageous or adaptive in any way? As described above, the lack of corpus and isthmus/TB coupling appeared to be useful for responding to changes in food density. Additionally, in the Rhabditidae family, the

expansion of pumping to the anterior isthmus is probably also functionally important, as computational modeling demonstrates that anterior isthmus pumping can drastically enhance the efficiency of food transport in the corpus (Avery and Shtonda 2003).

On the other hand, the other changes in pharyngeal behaviors did not have obvious functional consequence, or even appeared paradoxical. For example, in the Rhabditidae family, why TB pumping become coupled to corpus pumping is somewhat perplexing, since this prevented the ability to independently regulate corpus and TB pumping rates in response to changes in food density. One possibility might be that Rhabditidae species tend to favor food-rich localities, such as animal dung piles (personal communication, Michael Herman), such that dilute food conditions are typically not a factor. Or, it might be that coupling of the TB is an undesired, but necessary consequence of having gap junctions between the corpus and isthmus, in order to couple anterior isthmus pumping with the corpus. 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 (Zhang and Baldwin 1999; von Lieven and Sudhaus 2000), 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).

One way to understand how some of these behavioral differences might be adaptive, is to find differentially favored conditions for the different patterns of feeding behaviors (e.g. conditions A and B, where some families feed better in A, but other families feed better in B). Thus far, I may have found conditions that are universally favored by all free-living soil nematodes (preliminary experiments showed that all species favor smaller sized bacteria). In

contrast, I have yet to find any sets of differentially favored conditions (although my results above suggested that Diplogasteridae, Cephalobidae, and Panagrolaimidae species may be better adapted for sparse food conditions, compared to the Rhabditidae). In the future, it may be of interest to systematically screen for such differentially favored conditions.

Materials and Methods

Strains

Species from four free-living soil nematode families were examined: in family Panagrolaimidae – *Panagrolaimus sp.* PS1159, *Panagrolaimus sp.* PS1732, and *Panagrellus redivivus* DA1711 (derived by Leon Avery after 28x 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; and in family Rhabditidae – *C. elegans* N2, *Oscheius myriophila* DF5020, *Rhabditis blumi* DF5010, *Mesorhabditis longespiculosa* DF5017, *Teratorhabditis palmarum* DF5019, *Pelodera strongyloides* DF5013, *Poikilolaimus regenfussi* SB199. Information for these strains can be obtained on the internet at the Worm Systematics Resource Network (http://www.nyu.edu/projects/fitch/WSRN/) hosted by David Fitch. Many of these strains are also available from the Caenorhabditis Genetics Center (http://biosci.umn.edu/CGC/CGChomepage.htm). Taxonomic rankings for nematode groups are still frequently revised (De Ley and Blaxter 2002); thus, although I discuss these free-living soil nematode groups as "families", I caution that in the future their taxonomic ranks may be different. All of the above strains were maintained at 19°C on NGMSR plates

(Davis, Somerville et al. 1995) seeded with *E. coli* HB101 (Boyer and Roulland-Dussoix 1969), except DWF1301, PS1146, PS1197, DF5017, and DF5019, which were maintained at 25°C.

In addition, I also examined *Teratocephalus lirellus* PDL0011 as an outgroup species to the above four nematode families. *T. lirellus* had been similarly used as an outgroup to these families in other studies as well (such as in Zhang and Baldwin 2001). PDL0011 was maintained at 19°C on soil agar plates (1% agar, 1% fresh soil, 5μg/ml cholesterol) seeded with HB101.

Visual observations of feeding motions in free-living soil nematode species

I observed pharyngeal behaviors in all of the above species as follows. Bacterial suspensions were made by scraping *E. coli* from 1-2 seeded HB101 plates into 100μl of M9, followed by thorough mixing. Animals were then transferred to 2-3μl of the bacterial suspension, placed either on an unseeded NGMSR plate or a ~1mm thick NGMSR pad, and coverslipped. I typically waited ~30-60 minutes before making observations because feeding motions were often erratic and inconsistent immediately after coverslipping, presumably because the animals were initially disturbed by the protocol (this problem was more pronounced in some species.). Feeding motions were then observed using a Zeiss Axiophot microscope using 40x, 63x, or 100x objectives. Videos were taken with a Hitachi KP-160 CCD camera and digitized using Adobe Premiere v6.5. Compression of videos into MPEG files were done via SmartVideoConverter v1.5.15 (DoEase Software). Adults and larvae of each species generally displayed similar pharyngeal behaviors. The sample videos listed in

appendix A were taken from larvae, since their video quality is better than videos from adults, as smaller sizes permit better focusing on the pharyngeal lumen and easier tracking of moving animals.

Food density dependence assays

Normal bacterial suspensions were prepared as above (i.e. 1-2 HB101 plates/100µl M9), whereas *dilute* bacterial suspensions were prepared by diluting the normal suspensions 50-fold, except that for *Panagrolaimus sp.* PS1159 it was 20-fold. (PS1159 fed inconsistently or not at all when placed in 50-fold or more dilute suspensions.) I then coverslipped each animal in both normal and dilute bacterial suspensions and counted the pumping and peristalsis rates.

PS1159 and DWF1301 animals were often hyperactive and hard to track under the microscope specifically in the dilute condition. Roaming behaviors in *C. elegans* can be reduced if they are grown on poor quality food (Boris Shtonda, personal communication), such as the *E. coli* strain DA837 (Davis, Somerville et al. 1995). I 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/peristalses rates reported in Fig. 4 for PS1159 and DWF1301 animals are from DA837 preconditioned animals, to ensure consistency in the comparisons. For PS312, DA837 preconditioning was not necessary.

Further, *C. elegans* usually moves quite fast for accurate determination of peristalses rates in general (i.e. both normal and dilute conditions). Thus, instead of wild type animals, I

used slower moving *unc-29(e1072)* mutants, to obtain accurate measurements of posterior isthmus peristalsis rates. Although *unc-29* is not known to have significant effects on feeding, I independently confirmed the food density dependency of posterior isthmus peristalses rates by testing it in a different way, where wild type N2 larvae were placed in normal and dilute HB101 suspensions that also contained 10mM serotonin. The serotonin slowed down the animals sufficiently to allow accurate assessment of peristalsis rates, and I similarly found peristalses rates to be higher in the normal bacterial density than the dilute conditions (data not shown—qualitative results). The results shown in Figure 4 for *C. elegans* are derived from the *unc-29* experiments.

Figure 1 – General anatomy and behaviors of the *C. elegans* pharynx

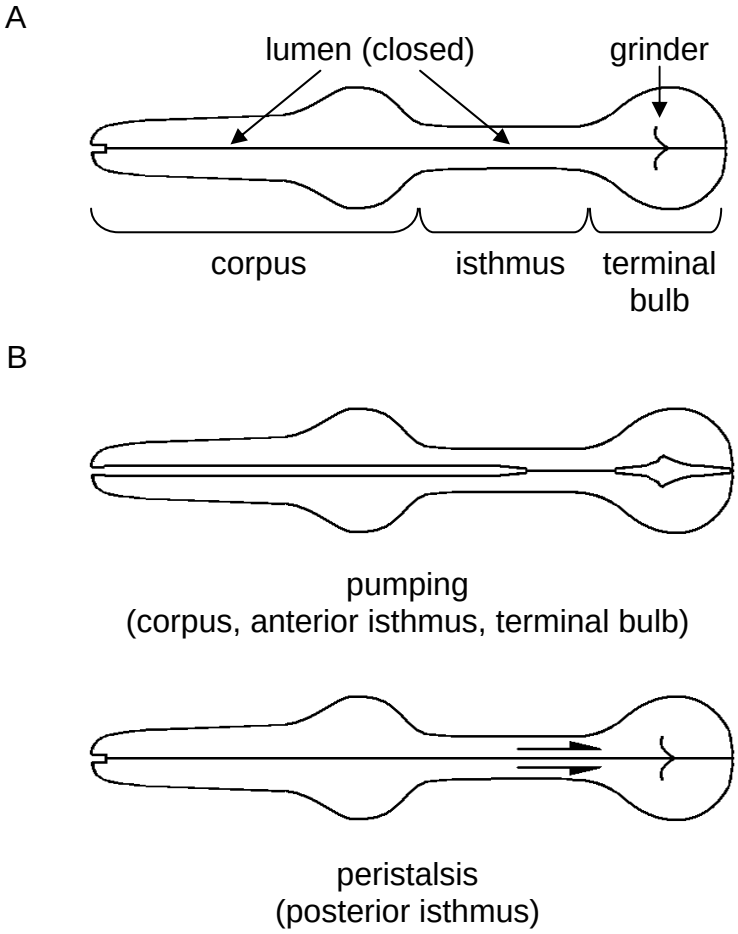

Figure 1 – General anatomy and behaviors of the *C. elegans* pharynx

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 is closed as shown.

B) Pumps are single, distinct muscle contractions that open the pharyngeal lumen, and pumping occurs in the corpus, anterior isthmus, and terminal bulb in *C. elegans* (indicated by the open lumen). Corpus, anterior isthmus, and terminal bulb pumping are all coupled – such that, with each pump, the corpus, anterior isthmus, and terminal bulb all contract together. Peristalses are posteriorly moving contraction waves, and occur in the posterior isthmus in *C. elegans* (indicated by the arrows). Posterior isthmus peristalses occur independently of corpus/anterior isthmus/terminal bulb pumping.

Figure 2 – Patterns of pharyngeal behaviors in free-living soil nematodes

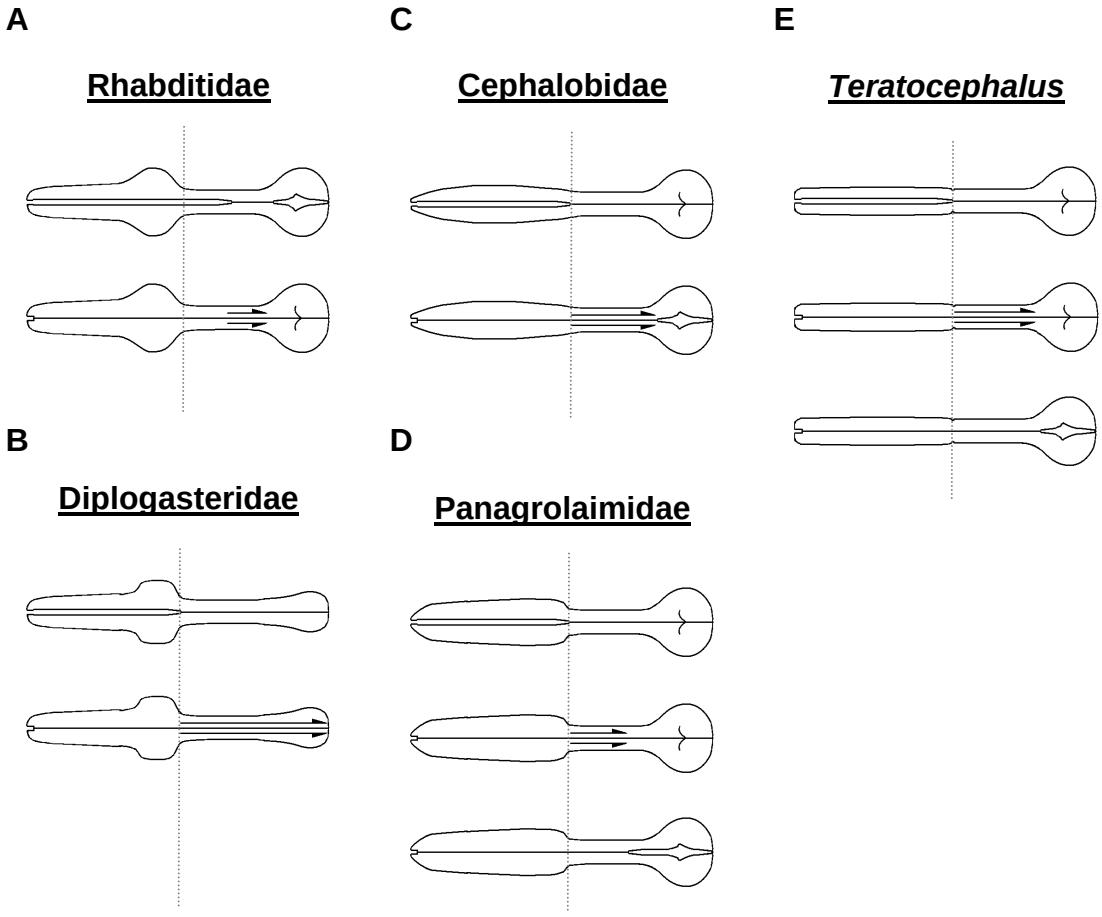

Figure 2 – Patterns of pharyngeal behaviors in free-living soil nematodes

Pharyngeal behaviors were observed in four free-living soil nematode families and an outgroup species (*Teratocephalus lirellus*). The behaviors are illustrated in A-E. Pumping is represented by open pharyngeal lumens and peristalsis is represented by arrows. 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 behaviors in the isthmus/TB.

A) Rhabditidae (including *C. elegans*): Pumping occurred anteriorly in the corpus and anterior isthmus, and posteriorly in the terminal bulb. Peristalsis occurred in the posterior isthmus. Corpus, anterior isthmus (AI), and TB pumping were all coupled. Posterior isthmus peristalses occurred independently.

B) Diplogasteridae: Pumping occurred in the corpus, and peristalses occurred throughout the entire isthmus and TB. Corpus pumping and isthmus/terminal bulb pumping occurred independently.

C) Cephalobidae: Pumping occurred anteriorly in the corpus and posteriorly in the terminal bulb. Peristalsis occurred in the entire isthmus. Isthmus peristalses and terminal bulb pumping were coupled, with every isthmus peristalsis accompanied by a TB pump.

D) Panagrolaimidae: Pumping occurred anteriorly in the corpus and posteriorly in the posterior isthmus and terminal bulb. Peristalses occurred in the anterior isthmus. Corpus pumping, AI peristalsis, and PI/TB pumping all occurred independently.

E) *T. lirellus*: Pumping occurred anteriorly in the corpus and posteriorly in the TB. Peristalsis occurred over the entire isthmus. Corpus pumping, isthmus peristalsis, and TB pumping all occurred independently.

Figure 3 – Model of pharyngeal behavior evolution in free-living soil nematodes

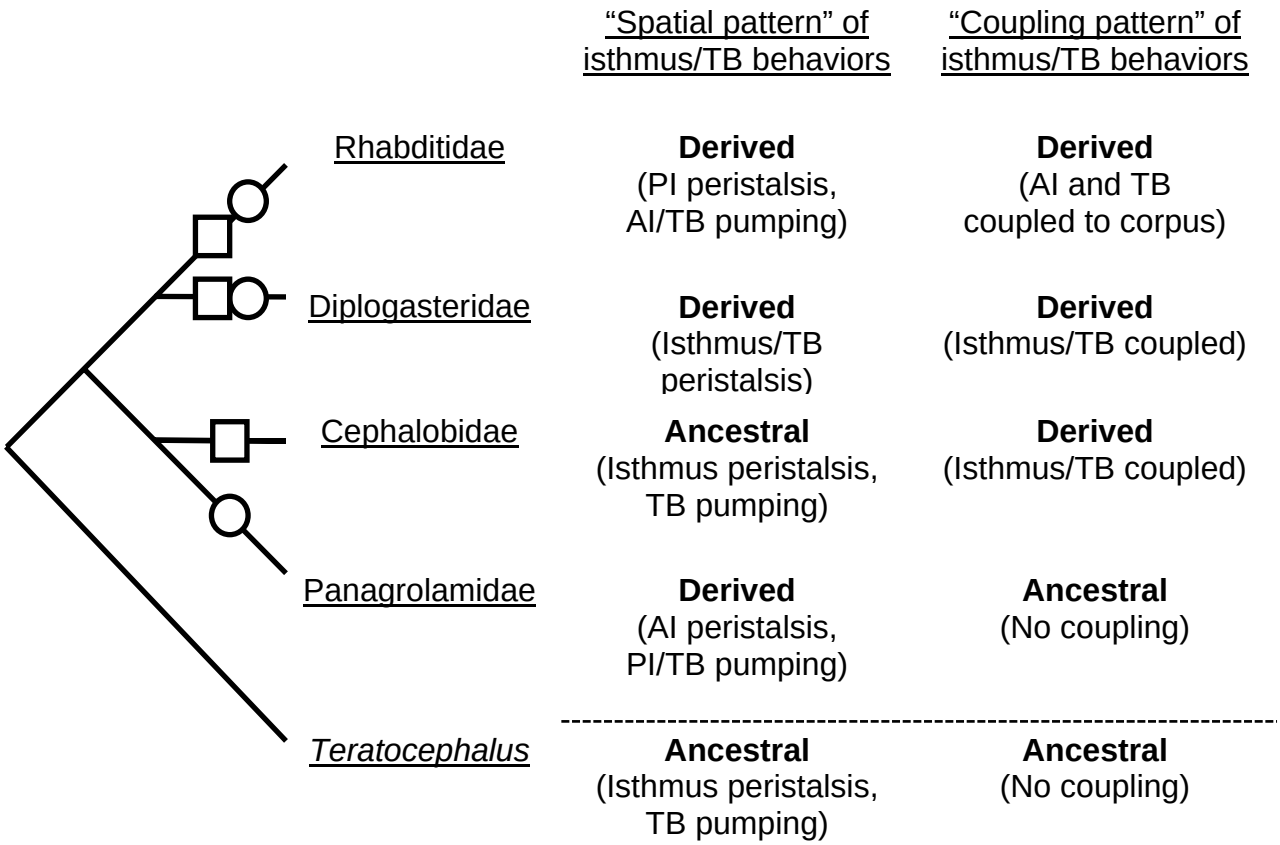

Figure 3 – Model of pharyngeal behavior evolution in free-living soil nematodes

A model of how pharyngeal behaviors evolved in free-living soil nematodes, together with their currently accepted phylogenetic relationships. "Spatial pattern" refers to where pumping and peristalsis occurred in the isthmus and TB, whereas "coupling pattern" refers to how isthmus/TB motions were coupled to each other or to the corpus.

Based on the outgroup species, *T. lirellus*, in the ancestor of free-living soil nematodes, the pharynx had corpus pumping, isthmus peristalses, and terminal bulb pumping, each of which occurred independently. In the Rhabditidae family, the anterior isthmus switched to pumping, and anterior isthmus and terminal bulb pumping became coupled to corpus pumping. In the Diplogasteridae family, the terminal bulb switched to peristalses. In the Cephalobidae family, isthmus peristalses and terminal bulb pumping became coupled. And in the Panagrolaimidae family, the posterior isthmus switched to pumping. Overall, the "spatial" pattern in the Cephalobidae is ancestral, whereas the "coupling" pattern in the Panagrolaimidae is ancestral. Open squares indicate when the "coupling" pattern evolved, and open circles indicate when the "spatial" pattern evolved.

Figure 4 – Decreased isthmus/terminal bulb contractions when food density is diluted

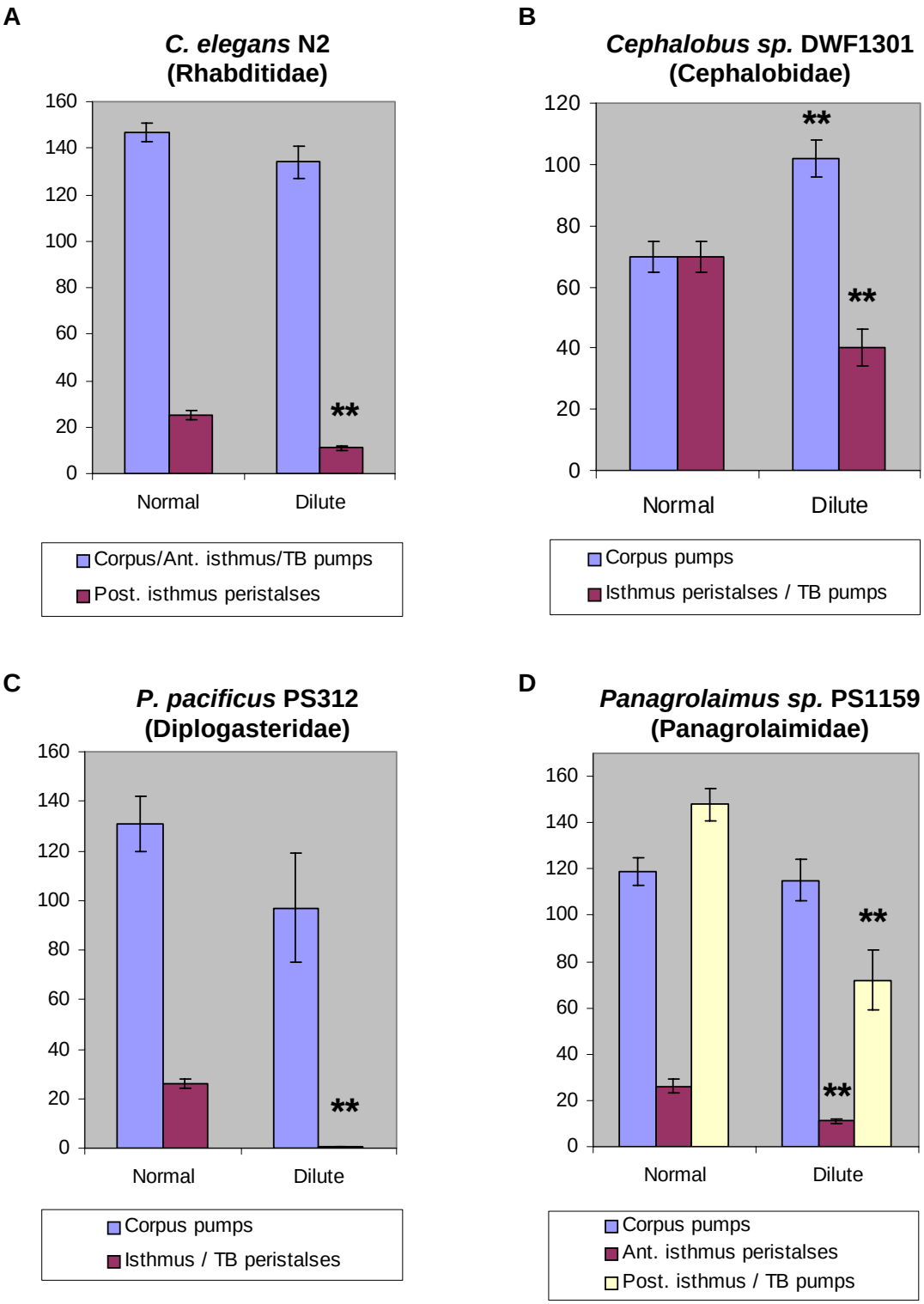

Figure 4 – Decreased isthmus/terminal bulb contractions when food density is diluted

A general feature of pharyngeal behaviors in free-living soil nematodes is that the corpus pumps independently of isthmus and/or terminal bulb contractions (see Figure 2), allowing the rates of corpus pumping and the rates of isthmus/TB contractions to be regulated independently. In species from each family, I found when food density is diluted (see Methods for details), corpus pumping in general remained largely similar or increased, whereas isthmus/terminal bulb peristalses/pumping rates decreased. This suggested that the lack of corpus and isthmus/TB coupling allowed useful independent regulation of their respective rates, in response to changes in food density.

A) In *C. elegans* (Rhabditidae), corpus/AI/TB pumping was similar in dilute food conditions, whereas posterior isthmus peristalsis was reduced.
B) In *P. pacificus* (Diplogasteridae), corpus pumping was similar in dilute food conditions, whereas isthmus/TB peristalsis was completely absent in dilute food conditions.
C) In *Cephalobus sp.* DWF1301 (Cephalobidae), corpus pumping increased in dilute food conditions, whereas isthmus peristalsis/TB pumping was reduced.
D) In *Panagrolaimus sp.* PS1159 (Panagrolaimidae), corpus pumping was similar in dilute food conditions, whereas anterior isthmus peristalses and posterior isthmus/TB pumping were decreased.

Asterisks indicate significant differences in the “dilute” condition, compared to the “normal” condition by two tailed T-test (** indicates $P<0.01$).

CHAPTER THREE

Evolution of M4/M2 function

& isthmus/TB stimulation

Introduction

Differences in isthmus and TB behaviors suggested differences in isthmus and TB stimulation

Having observed significant differences in isthmus/TB behaviors, I next wished to investigate what changes in neuronal regulation may have corresponded with and contributed to these behavioral differences. In *C. elegans*, pharyngeal behaviors are regulated by a self-contained pharyngeal nervous system. In total, there are 14 types of *C. elegans* pharyngeal neurons (Albertson and Thomson 1976), but the only known excitatory motor neurons are (schematized in Fig. 5): M4, which is required for posterior isthmus peristalsis (Avery and Horvitz 1987) and MC, which directly innervates and stimulates corpus pumping (Avery and Horvitz 1989; Raizen, Lee et al. 1995; McKay, Raizen et al. 2004) and indirectly stimulates AI and TB to pump via gap junctions located between the corpus and the isthmus (Starich, Lee et al. 1996 and unpublished observations). Thus, the two major sources of isthmus and TB excitations in *C. elegans* are: 1) M4 and 2) electrical coupling to the corpus.

How might stimulation of isthmus and TB contractions compare in the Diplogasteridae, Cephalobidae, and Panagrolaimidae families? The changes in isthmus and TB behaviors suggested that differences in isthmus and TB stimulation probably evolved as well. First, since corpus pumping and isthmus/TB motions were not coupled in these three families, electrical coupling to the corpus is apparently not as significant. On the other hand, the nervous systems of nematodes are generally well conserved anatomically (Martin, Purcell et al. 2002), and I was similarly able to identify homologs of most *C. elegans* pharyngeal neurons in other free-living soil nematodes (see Figure 6—note that I could not identify TB

neurons in the Diplogasteridae, and identifications were also more tentative for ventral corpus neurons in general). Therefore, is M4 conserved as an excitatory neuron in the other families, and if so, has its function changed? Even if M4 functions similarly to stimulate peristalsis, its net effects must still have evolved, since peristalses in the other families do not occur in just the posterior isthmus. Further, do other pharyngeal neurons affect isthmus/TB behaviors in the other families? For instance, since electrical coupling to the corpus is less important in the Panagrolaimidae, another pharyngeal neuron may be involved in stimulating PI/TB pumping.

M4 and M2 are key excitatory neurons for the isthmus and terminal bulb in *Panagrellus redivivus*

Mark Steciuk, another student in the lab, examined pharyngeal neuron functions in one species from the Panagrolaimidae family: *Panagrellus redivivus*. By individually ablating each neuronal type in *P. redivivus* and examining the resulting phenotypes, he found that in contrast to *C. elegans*, the key *P. redivivus* excitatory neurons were MC, M4, and M2. MC was conserved with *C. elegans* in its function to stimulate corpus pumping, whereas M4 diverged in function, and instead stimulated PI/TB pumping in *P. redivivus*. Further, M2, a neuron which has no obvious function in *C. elegans*, was important for anterior isthmus peristalsis. Thus, Mark showed that M4 and M2 functioned rather differently in *P. redivivus*, and resulted in a significantly different pattern of isthmus and TB stimulation, compared to *C. elegans*.

Results

M4 stimulated different isthmus/TB behaviors in each free-living soil nematode family, whereas M2 only stimulated anterior isthmus peristalsis in the Panagrolaimidae

Based on the differences in M4 and M2 function in *P. redivivus,* I decided to investigate the functions of these two neurons more broadly in free-living soil nematodes, to further understand how M4 and M2 functions evolved in general, and to investigate other potential differences in isthmus and TB stimulation.

First, the Rhabditidae family is extremely diverse, and has many more known species than the other three families (Blaxter, De Ley et al. 1998). Thus, are M4/M2 functions conserved between *C. elegans* and other members of this expansive family? *Mesorhabditis longespiculosa* is relatively far diverged from *C. elegans* within the Rhabditidae family (D. Fitch, personal communication). I found that while ablating M2 in *M. longespiculosa* caused no obvious phenotype, killing M4 caused a loss of posterior isthmus peristalsis (Fig. 7A) which sometimes also caused the anterior isthmus and corpus to become stuffed with bacteria (Fig. 8A). The effects of killing M2 and M4 in *M. longespiculosa* were thus virtually identical to those of killing M2 and M4 in *C. elegans* (Avery and Horvitz 1987; Avery and Horvitz 1989), indicating that the two neurons are functionally conserved within the large Rhabditidae family.

Then, since M2 and M4 functions in the Diplogasteridae and Cephalobidae families had not been examined, I continued with laser ablations in species from these two families. In the Diplogasteridae, the homologue of M2 could not be readily identified (see Fig. 6), so only M4 ablation was possible. In *Pristionchus pacificus,* a Diplogasteridae species, killing

M4 caused a large reduction in isthmus/TB peristalsis (Fig. 7B), which also caused their corpuses to become stuffed with bacteria (Fig. 8B), similar to M4-ablated *C. elegans*. Thus, M4 in the Diplogasteridae (*P. pacificus*) also served to stimulate peristalsis, similar to M4 in the Rhabditidae. At the same time, M4 function in the Diplogasteridae was different from the Rhabditidae as well, in that it stimulated peristalses in the entire isthmus and TB, and not just in the posterior isthmus.

In *Cephalobus sp.* DWF1301, a Cephalobidae species, ablation of M2 caused no apparent phenotype. Ablation of M4, however, caused a decrease in isthmus peristalsis/TB pumping (Fig. 7C). Thus, similar to the Rhabditidae and the Diplogasteridae, M4 was an important excitatory neuron in the Cephalobidae. The exact function of M4 in the Cephalobidae, however, differed from those families, in that M4 stimulated both (isthmus) peristalsis and (terminal bulb) pumping.

And finally, I wished to examine M2 and M4 functions in another Panagrolaimidae species, since the pharyngeal behaviors of *P. redivivus* were somewhat different from the other Panagrolaimidae species I observed (in *P. redivivus*, some PI/TB pumps were coupled to corpus pumps in *P. redivivus*, whereas in other Panagrolaimidae species, PI/TB pumps were entirely independent of corpus pumping). I found that M2/M4 ablation in *Panagrolaimus sp.* PS1159 caused essentially the same phenotypes as in *P. redivivus*, where killing M4 caused a specific reduction in PI/TB pumping (Fig. 7D), and killing M2 caused a dramatic decrease in AI peristalsis (Fig. 7D). Interestingly, using electropharyngeograms (see Materials and Methods) to analyze the temporal characteristics of corpus and PI/TB pumping, I found that after M4 ablation, all the remaining PI/TB pumps in *Panagrolaimus*

sp. PS1159 were coupled to corpus pumps (Fig. 9). This suggested that while electrical coupling to the corpus was not normally important in *Panagrolaimus sp.* PS1159, it could serve as a backup mechanism for stimulating PI/TB pumping in the absence of M4 function.

Altogether, while isthmus and TB behaviors varied greatly in free-living soil nematodes, M4 was a generally important excitatory neuron in all four families. The exact function of M4 varied, however, where M4 stimulated PI peristalsis in the Rhabditidae, isthmus/TB peristalsis in the Diplogasteridae, isthmus peristalsis/TB pumping in Cephalobidae, and PI/TB pumping in Panagrolaimidae. Further, M4 alone did not account for all of the sources of isthmus/TB excitation in free-living soil nematodes. In the Rhabditidae, electrical coupling to the corpus is critical for stimulating AI/TB pumping. And, in the Panagrolaimidae, M2 stimulated AI peristalsis, and electrical coupling to the corpus contributed to PI/TB pumping as well. Schematics of the different patterns of isthmus and TB stimulation in each family are shown in Fig. 10, and a table that summarizes M4 and M2 functions is shown in Fig. 11.

Regulation of isthmus/TB contractions in response to food density changes requires M4

Since M4 was a key excitatory neuron for isthmus/TB contractions, and the rates of isthmus/TB contractions were generally regulated in response to food density changes (see Chapter 2), I directly tested if M4 was required for the food density responses. In M4 ablated animals (Fig. 12A-C), I found that the rates of the corresponding isthmus/TB contractions were largely similar to control animals in the dilute food conditions, but drastically lower in the normal food conditions (note that the isthmus/TB rates of control and M4- animals

measured at normal food densities were in essence the same results as in Fig. 7). Thus, M4 was important for the food density response, where higher food density activated M4 to stimulate corresponding isthmus/TB contractions.

Discussion

Evolution of isthmus and TB stimulation by the M2 and M4 neurons

The differences in isthmus and terminal bulb behaviors suggested changes in the neuronal stimulation and regulation of these behaviors. Based on Mark Steciuk's initial observations in *P. redivivus*, I conducted M4 and M2 laser ablations in additional species from each free-living soil nematode family, to broadly investigate their functions in the isthmus and terminal bulb. Overall, whereas M2 functioned specifically in the Panagrolaimidae to stimulate anterior isthmus peristalsis, I found M4 to be a generally important excitatory neuron for isthmus and TB behaviors, whose exact downstream function varied in each family.

Overall, M4's downstream function on pharyngeal muscles varied in two different ways. With respect to the pharyngeal regions affected by M4 stimulation, I found that M4 stimulated only the posterior isthmus (PI) in the Rhabditidae, both PI and TB in the Panagrolaimidae, or the entire isthmus and TB in the Diplogasteridae and Cephalobidae. With respect to pumping and peristalsis, M4 stimulated only peristalsis in the Rhabditidae and Diplogasteridae, only pumping in the Panagrolaimidae, and both pumping and peristalses in the Cephalobidae. So, what might be the underlying mechanisms responsible for these changes in M4 function? Some possibilities are suggested by the analyses of *slo-1* and *ceh-*

28 mutants in *C. elegans*, which are described in Chapters 4 and 5 below. Also, note that the underlying differences need not be in M4 *per se*, but could actually be in the downstream muscles, with respect to how they respond to M4 stimulation.

While M4's downstream functions evolved between the four families, I also found that M4 responded generally to alterations in food density. What exactly triggers M4 activity when food density is increased? I suggest two nonmutually exclusive possibilities: 1) M4 responds to the presence and amount of food ingested into the corpus (e.g. by direct mechanosensation in the corpus), or 2) M4 responds to the nutritional/satiation status of the animal (e.g. by a neurohumoral factor from the gut).

While these results provided a general picture of how neuronal stimulation of isthmus and terminal bulb behaviors evolved in free-living soil nematodes, there are likely still additional mechanisms of isthmus/TB stimulation to be identified. For example, killing M4 in the Cephalobidae (*Cephalobus sp.* DWF1301) only reduced, but did not eliminate, isthmus/TB pumping. Also, killing M4 in the Diplogasteridae (*P. pacificus*) did not entirely eliminate isthmus/TB peristalsis. In these cases, either other pharyngeal neurons can also stimulate the corresponding isthmus/TB contractions, or the muscles themselves are capable of generating the contractions myogenically without additional neuronal input. In the Cephalobidae, these other mechanism(s) for stimulating isthmus peristalses/TB pumping appeared to be especially significant, as ablating M4 only reduced isthmus peristalsis/TB pumping by ~1/3.

Further, although I attempted laser ablations in the outgroup *T. lirellus* to try to infer the ancestral functions of M4 and M2, the ablations were unsuccessful due to technical

difficulties in growing and handling *T. lirellus* animals. Accurate staging of the age of *T. lirellus* larvae, important for the laser ablation protocol, was not possible. Single worm cultures of *T. lirellus* animals, important for keeping track of operated animals after ablations, were also not possible. Thus, I was not able to infer the ancestral functions of the M4 and M2 neurons.

Gap junctions between the corpus and isthmus in the Rhabditidae

Based on the behavioral differences, I have largely assumed without direct proof that electrical coupling due to gap junctions between the corpus and the isthmus/TB is significant in the Rhabditidae, but less significant or entirely absent in the Diplogasteridae, Cephalobidae, and Panagrolaimidae families. Some preliminary electrophysiological measurements in the Panagrolaimidae family (Boris Shtonda, personal communication) are consistent with this assumption, where intracellular recordings of the TB showed that TB membrane potentials are minimally affected by corpus pumping.

The *C. elegans* innexin subunit encoded by *eat-5* is necessary for the function of gap junctions between the corpus and isthmus (Starich, Lee et al. 1996). Thus, specific differences in the *eat-5* gene may have contributed to how these gap junctions evolved. For example, *eat-5* may have been a novel gene that arose specifically in the Rhabditidae family, or *eat-5* might be decreased in expression or function in the Diplogasteridae, Cephalobidae, and Panagrolaimidae families.

Note concerning the neuronal stimulation of corpus pumping

In addition to the isthmus/terminal bulb phenotypes, killing M4 in *P. pacificus* PS312 and M2 in *Panagrolaimus sp.* PS1159 also led to moderate decreases in corpus pumping (Fig. 7B, D). While these results might suggest that M4 and M2 also stimulate corpus pumping in the Diplogasteridae and Panagrolaimidae, I think that they are actually secondary to the isthmus/TB peristalsis defects caused by M4/M2 ablation. In *C. elegans*, defective peristalsis in M4 ablated animals also reduces corpus pumping, due to the accumulation of bacteria in the corpus (Raizen, Lee et al. 1995), and I think a similar mechanism may be at work in M4/M2 ablated *P. pacificus* and *Panagrolaimus sp.* PS1159 animals.

In general, MC is probably conserved in free-living soil nematodes as the key neuron for stimulating pumping in the corpus. This statement is largely based on Mark's observations that MC is conserved in function between *P. redivivus* and *C. elegans* (Mark Steciuk, personal communication), and the fact that these two species are as distantly related as free-living soil nematodes can be (Blaxter, De Ley et al. 1998). Further, my observations in Chapter 2 that corpus behaviors are largely conserved also correlates well with the notion that neuronal regulation of corpus pumping by MC is overall conserved.

Materials and Methods

Laser ablations in free-living soil nematodes

I chose *M. longespiculosa* DF5017, *P. pacificus* PS312, *Cephalobus sp.* DWF1301, and *Panagrolaimus sp. PS1159* 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 hours old as previously described for *C. elegans* (Bargmann and Avery,

1995), except for the anesthetic conditions. For *P. pacificus* PS312, the larvae were anesthetized on 10mM NaN_3 pads, as in *C. elegans*. For *Panagrolaimus sp.* PS1159 and *M. longespiculosa* DF5017, the larvae were anesthetized on 20mM NaN_3 pads. For *Cephalobus sp.* DWF1301, larvae were placed in ~1µl of 100mM NaN_3 on top of 10mM NaN_3 pads for ~30 seconds until the animals relaxed by curling up, and then coverslipped. I assayed the pumping and peristalsis rates of operated animals after they reached adulthood, determined by either the presence of oocytes or mating spicules (*M. longespiculosa* DF5017), or the presence of laid eggs on the plate (all the other species). Rates of pumping and peristalses were determined by coverslipping the animals as described in Chapter 2, followed by counting the number of pumps/peristalses in one minute.

Two types of controls were performed to verify that the changes in pharyngeal function were specific to losses of M2/M4 function. I did: 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 neurons near M4 (i.e. M3 or NSM) and M2 (i.e. I6) were ablated, to control for nonspecific effects of laser damage. In general, the effects of mock and nearby ablations were indistinguishable, and the control results in Fig. 7A-C were derived from mock ablations. The only exception was *M. longespiculosa* DF5017, where nearby ablated worms appeared to have slightly decreased corpus pumping compared to mock ablated worms (by ~20 pumps/min), indicating that laser damage caused a general, nonspecific drop in pumping. Thus for *M. longespiculosa* DF5017, the control results in Fig. 7D represent values derived from nearby ablated animals.

Electropharyngeograms (EPGs) in *Panagrolaimus sp.* PS1159 animals

Electropharyngeograms on *Panagrolaimus sp.* PS1159 animals were performed on dissected, exposed pharynxes as previously described for *C. elegans* (Davis et al., 1995), except *Panagrolaimus sp.* PS1159 required higher concentrations of serotonin (5HT) than in *C. elegans* to consistently stimulate pumping. Thus, the EPG bath solution was modified to contain 0.9x Dent's saline with 10μM serotonin (5HT), which was made by mixing 9 parts Dent's saline with 1 part 100μM serotonin (5HT). 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).

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Figure 5 – Neuronal stimulation of pumping and peristalsis by the MC and M4 neurons in *C. elegans*

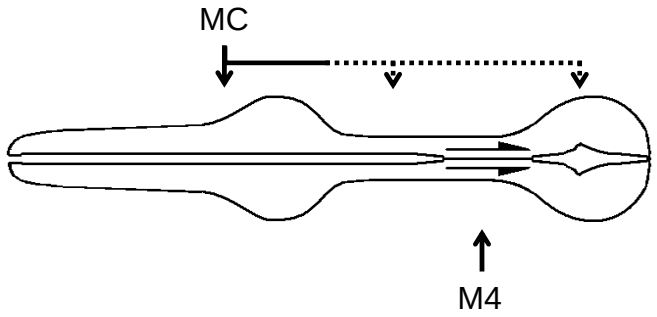
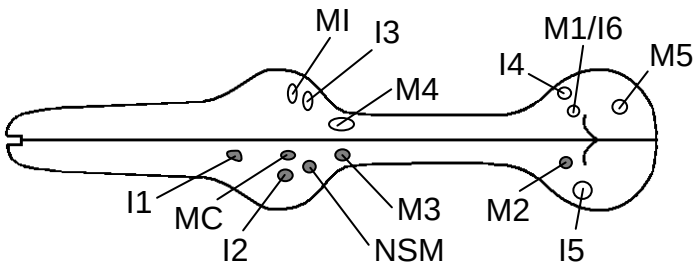


Figure 5 – Neuronal stimulation of pumping and peristalsis by the MC and M4 neurons in *C. elegans*

A schematic illustrating stimulation of pumping and peristalsis by M4 and MC, the two major excitatory pharyngeal neuron types in *C. elegans*. 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. The dashed arrows indicate the indirect stimulation of anterior isthmus and TB pumping.

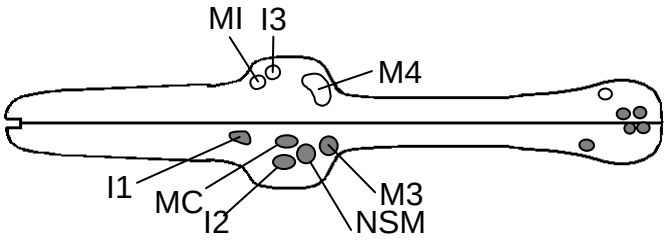
Figure 6 – Identification of pharyngeal neurons in free-living soil nematodes

A



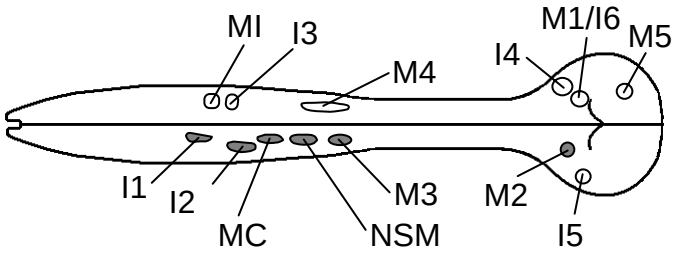
Caenorhabditis elegans (Rhabditidae)

B



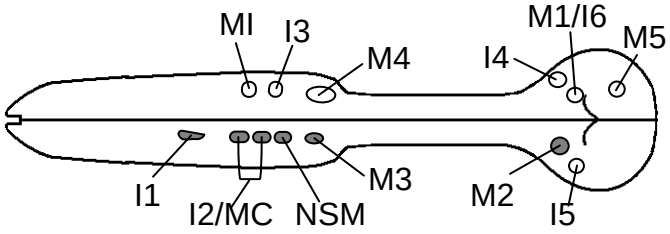
Pristionchus pacificus (Diplogasteridae)

C



Cephalobus sp. DWF1301 (Cephalobidae)

D



Panagrolaimus sp. PS1159 (Panagrolaimidae)

Figure 6 – Identification of pharyngeal neurons in free-living soil 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 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 homologues of *C. elegans* pharyngeal neurons in *Pristionchus pacificus* PS312 (Diplogasteridae), *Cephalobus sp.* DWF1301 (Cephalobidae), and *Panagrolaimus sp.* PS1159 (Panagrolaimidae). Most of the pharyngeal neuron types in *C. elegans* 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 generally more tentative, and I2 and MC could not be distinguished in several species.

Figure 7 – Effects of M2 and M4 ablation in select free-living soil nematode species

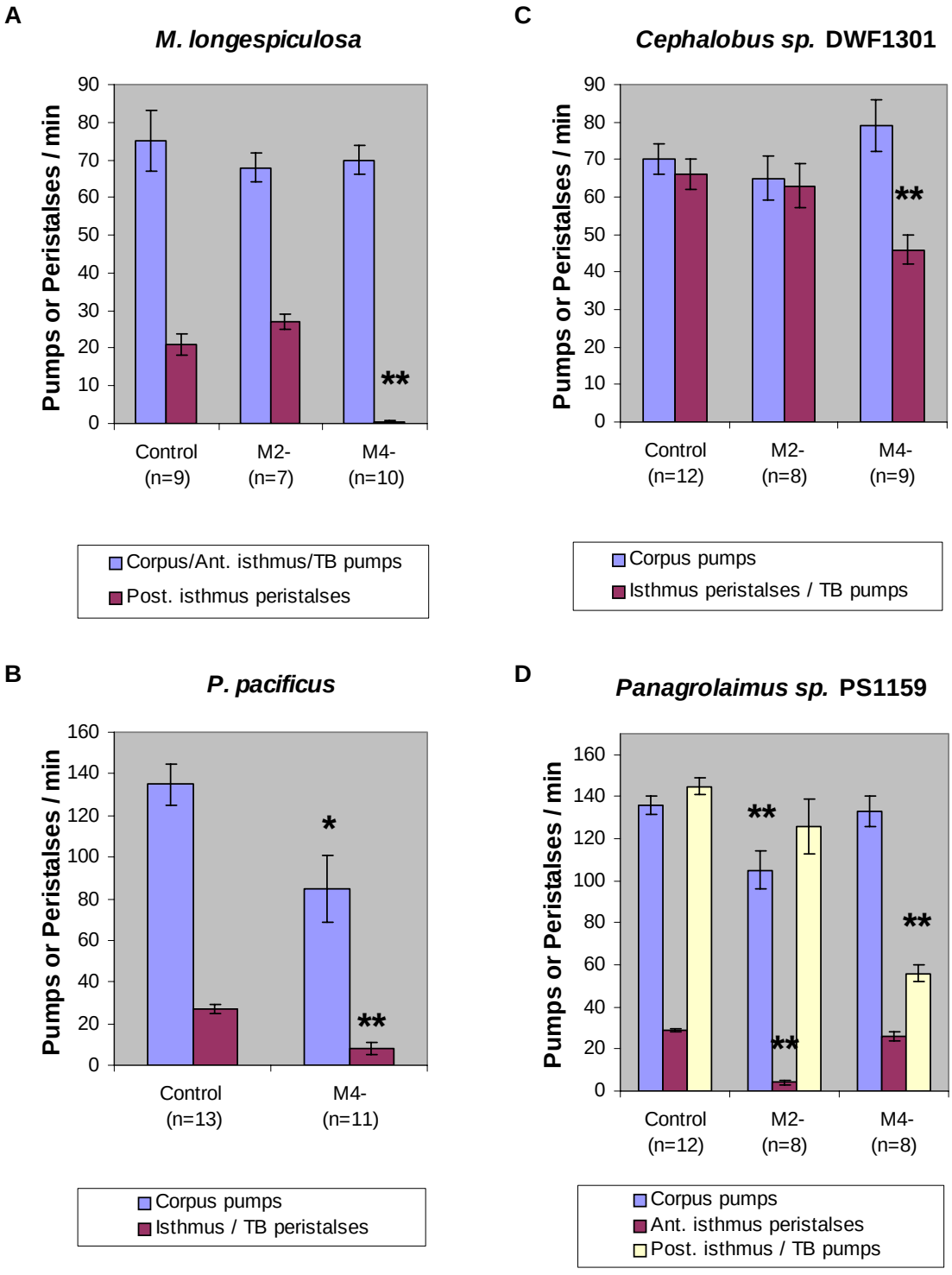


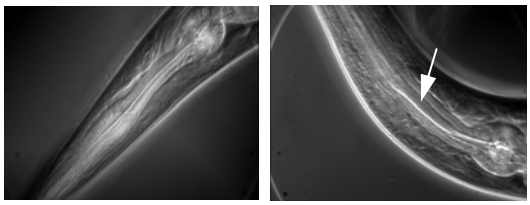
Figure 7 – Effects of M2 and M4 ablation in select free-living soil nematode species

The effects of M2 and M4 laser ablations in select free-living soil nematode species from each major family. Note that I think the effects on corpus pumping by ablating M4 in *P. pacificus* and M2 in *Panagrolaimus sp.* PS1159 are secondary to the defects in peristalses (see text). Asterisks indicate statistically significant differences from controls by two-tailed T-test (** for $p<0.01$, * for $p<0.05$).

A) *Mesorhabditis longespiculosa* DF5017 (Rhabditidae): M4 ablation eliminated posterior isthmus peristalsis, whereas M2 ablation caused no effects.
B) *Pristionchus pacificus* PS312 (Diplogasteridae): M4 ablation caused a large reduction in isthmus/TB peristalses, and a decrease in corpus pumping as well. M2 ablation was not done, since M2 could not be identified (see text and Fig. 6).
C) *Cephalobus sp.* DWF1301 (Cephalobidae): M4 ablation caused a reduction in isthmus peristalses/TB pumping, whereas M2 ablation caused no effects.
D) *Panagrolaimus sp.* PS1159 (Panagrolaimidae): M4 ablation caused a large reduction in posterior isthmus/TB pumping. M2 ablation caused a drastic reduction of anterior isthmus peristalses and a reduction of corpus pumping as well.

Figure 8 – M4 ablations caused stuffed pharynx phenotypes in *M. longespiculosa* DF5017 and *P. pacificus* PS312

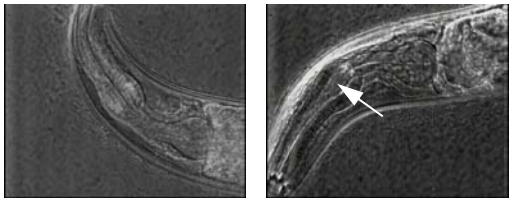
A



Mock M4-

M. longespiculosa DF5017
(Rhabditidae)

B



Mock M4-

P. pacificus PS312
(Diplogasteridae)

Figure 8 – M4 ablations caused stuffed pharynx phenotypes in *M. longespiculosa* DF5017 and *P. pacificus* PS312

As in M4-ablated *C. elegans*, peristalsis defects due to M4 ablation in *M. longespiculosa* DF5017 and *P. pacificus* PS312 animals caused the pharynx to become stuffed with bacteria, as indicated by the arrows.

A) Stuffed corpus/anterior isthmus in *M. longespiculosa* DF5017 animals.
B) Stuffed corpus in M4 ablated *P. pacificus* PS312 animals.

Figure 9 – Posterior isthmus/TB pumping is coupled to corpus pumping in M4 ablated *Panagrolaimus sp.* PS1159 animals

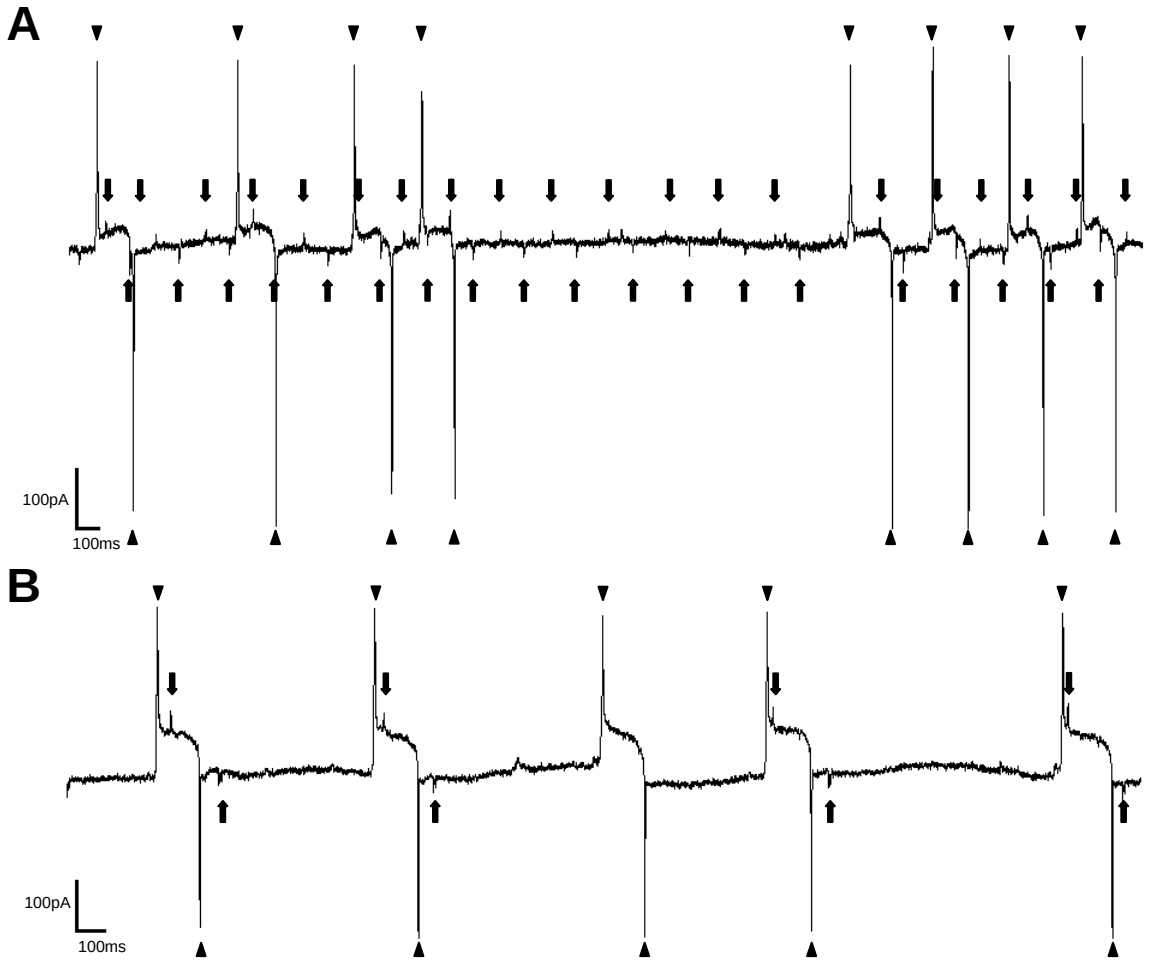


Figure 9 – Posterior isthmus/TB pumping is coupled to corpus pumping in M4 ablated *Panagrolaimus sp.* PS1159 animals

Electropharyngeograms, extracellular current recordings of the pharynx, were adapted for *Panagrolaimus sp.* PS1159 (see text). Each corpus pump generates a large positive and large negative spike, indicated by the arrowheads. Each PI/TB pump generates a small positive and small negative spike, indicated by the arrows.

A) In control (normal) *Panagrolaimus sp.* PS1159 animals, corpus and PI/TB pumps occurred independently.
B) In M4 ablated *Panagrolaimus sp.* PS1159 animals, PI/TB pumps occurred only immediately after corpus pumps, indicating coupling of PI/TB pumping to the corpus.

Figure 10 – Schematic of neuronal stimulation in the isthmus and terminal bulb of free-living soil nematodes

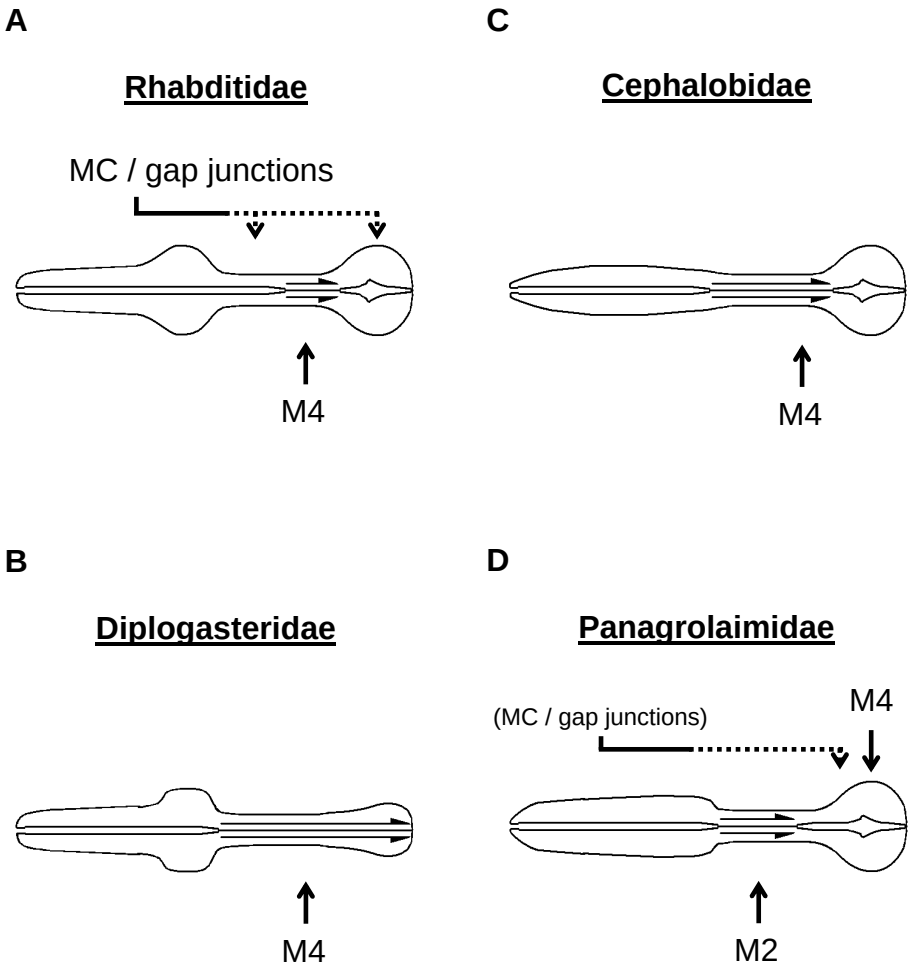
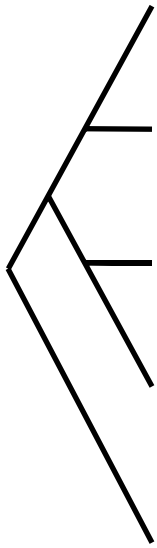


Figure 10 – Schematic of neuronal stimulation in the isthmus and terminal bulb of free-living soil nematodes

The major sources of neuronal stimulation of isthmus and terminal bulb (TB) behaviors, as revealed by laser ablations.

A) Rhabditidae – M4 stimulates posterior isthmus peristalses. Anterior isthmus/TB pumping is indirectly stimulated by MC via gap junction coupling to the corpus (dashed arrows).
B) Diplogasteridae – M4 stimulates isthmus/TB peristalses.
C) Cephalobidae – M4 stimulates isthmus peristalses and TB pumping.
D) Panagrolaimidae – M2 stimulates anterior isthmus peristalsis while M4 stimulates posterior isthmus/TB pumping. Gap junction coupling also contributes to stimulation of posterior isthmus/TB pumping (as a backup mechanism in *Panagrolaimus sp.* PS1159).

Figure 11 – Summary table of M4 and M2 functions, and gap junction coupling, in free-living soil nematodes



	M4	M2	Gap junction (corpus/isthmus)
Rhabditidae	Post. isthmus peristalsis	No function	Yes
Diplogasteridae	Isthmus/TB peristalsis	No function	No
Cephalobidae	Isthmus peristalsis/ TB pumping	No function	No
Panagrolamidae	Post. isthmus/TB pumping	Ant. isthmus peristalsis	Partial
Teratocephalus	?	?	No

Figure 11 – Summary table of M4 and M2 functions, and gap junction coupling, in free-living soil nematodes

M4 functions in all four families, where M4 stimulates posterior isthmus peristalses in the Rhabditidae, isthmus/TB peristalsis in the Diplogasteridae, isthmus peristalsis/TB pumping in the Cephalobidae, and posterior isthmus/TB pumping in the Panagrolaimidae. M2 functions only in the Panagrolaimidae to stimulate anterior isthmus peristalsis. Laser ablations of M4 and M2 in *T. lirellus* were not feasible, due to technical challenges (see text).

Gap junction coupling is important mostly in the Rhabditidae for stimulating anterior isthmus/TB pumping. However, it also plays a partial role in stimulating posterior isthmus/TB pumping in the Panagrolaimidae. Note that gap junction functions were inferred from the coupling or lack of coupling of pharyngeal behaviors (either on video microscopy or EPGs), i.e. direct measurements of gap junction functions were not made.

Figure 12 – M4 is activated by increased food density to stimulate isthmus/TB pumping/peristalses

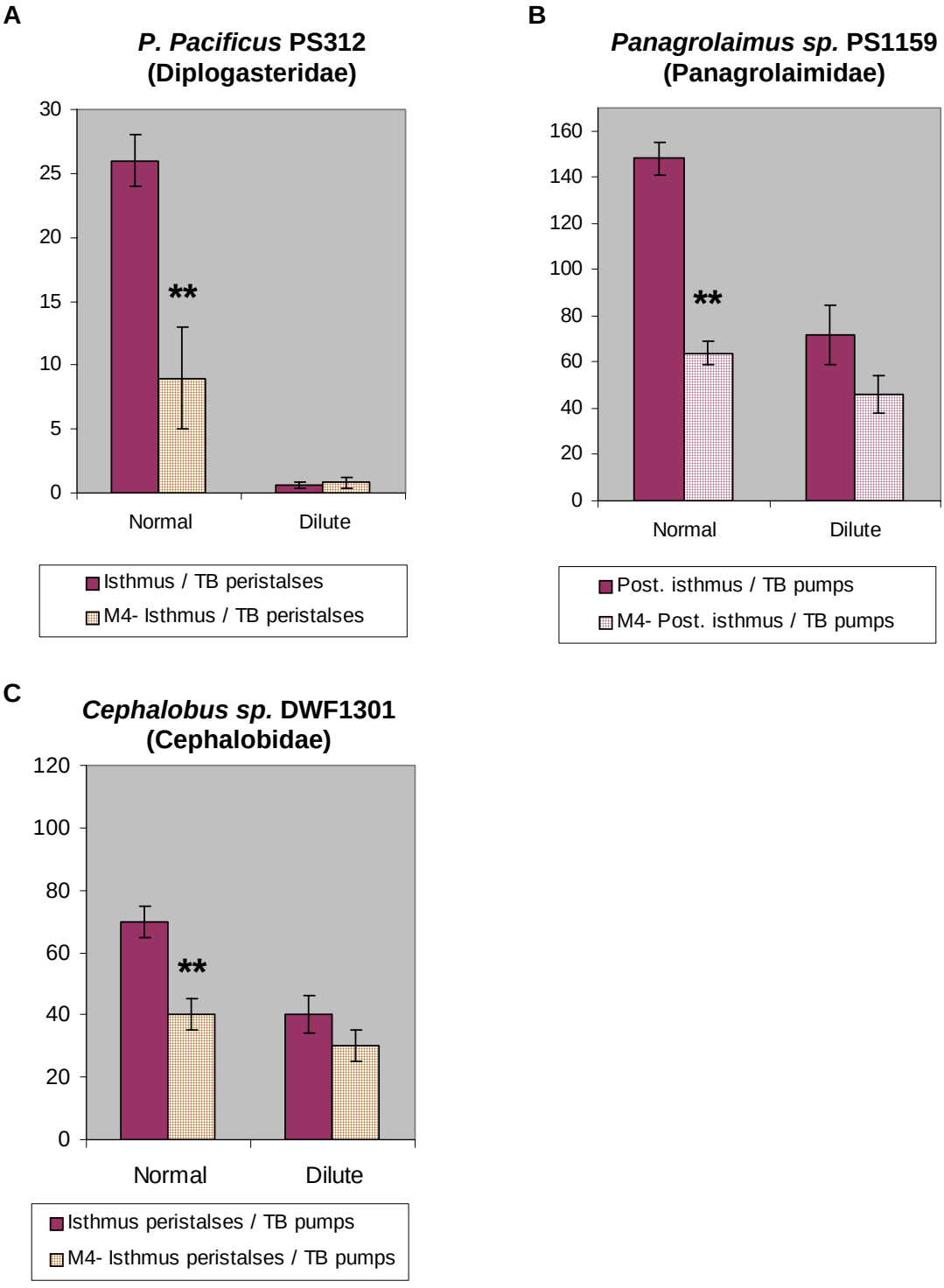


Figure 12 – M4 is activated by increased food density to stimulate isthmus/TB pumping/peristalses

In M4 ablated animals, the rates of corresponding isthmus/TB contractions were largely similar to control in the dilute food conditions, but drastically lower in the normal food conditions. Thus, M4 is important for the food density response, and acts as a food density sensor – i.e. higher food density activates M4 to upregulate corresponding isthmus/TB contractions. Asterisks indicate significant differences between M4 ablated and control animals by two tailed T-test (** for $P<0.01$).

A) *P. pacificus* PS312 – M4 ablated animals have similar isthmus/TB peristalses rates in dilute food conditions, but drastically lower isthmus/TB peristalses rates in normal food conditions.
B) *Cephalobus sp.* DWF1301 – M4 ablated animals have similar isthmus peristalses /TB pumping rates in dilute food conditions, but drastically lower isthmus peristalses /TB pumping rates in normal food conditions.
C) *Panagrolaimus sp.* PS1159 – M4 ablated animals have similar PI/TB pumping rates in dilute food conditions, but drastically PI/TB pumping rates in normal food conditions.

CHAPTER FOUR

Loss of *slo-1* activated M4-TB stimulations in *C. elegans*

Introduction

Why does M4 not stimulate the terminal bulb in *C. elegans* and the Rhabditidae family?

As shown in Chapter Three, the functions of M4 varied from family to family in free-living soil nematodes, including the exact pharyngeal regions stimulated by M4. One difference specific to the Rhabditidae family was that M4 had no effect on the terminal bulb, whereas in the other three families, M4 stimulation did affect the terminal bulb. Why might this be? Since *C. elegans* is a Rhabditidae species, I examined the relationship between M4 and the TB more carefully in *C. elegans*, to try to understand why M4-TB stimulations are lacking in the Rhabditidae family.

Results

M4 innervated TB muscles in *C. elegans*

One possible reason for the lack of M4-TB stimulations could be that M4 simply does not innervate TB muscles. Therefore, together with Boris Shtonda (another graduate student in the laboratory), I characterized the synaptic pattern of M4 in *C. elegans* using *Pceh-28::snb-1::gfp*, a transgene in which the *Pceh-28* promoter drives M4 specific expression of the synaptic vesicle marker SNB-1::GFP (Nonet 1999). In these transgenic animals, I found punctate clusters of SNB-1::GFP along M4 axons in both the posterior isthmus and terminal bulb (Fig. 13A), indicating that M4 did in fact innervate the terminal bulb in *C. elegans*. To confirm that the observed puncta truly corresponded to synapses, I also expressed *Pceh-28::snb-1::gfp* in *unc-104* mutants, in which loss of *unc-104* disrupts axonal transport of synaptic vesicles (Hall and Hedgecock 1991; Otsuka, Jeyaprakash et al. 1991), and SNB-

1::GFP that is normally synaptically localized should instead accumulate in cell bodies (Nonet, Saifee et al. 1998; Nonet 1999). Indeed, in *unc-104* mutants that harbored the *Pceh-28::snb-1::gfp* transgene, I found that SNB-1::GFP mislocalized to the M4 cell body, and the previously observed puncta in the isthmus and TB were absent (Fig. 13B).

Loss of *slo-1* function activated *C. elegans* M4-TB synapses

Thus, the lack of M4-TB stimulations did not appear to be from a lack of innervation. Rather, M4-TB synapses are present, but are somehow not functional – thus, what might be the reason? The gene *slo-1* encodes a BK type Ca^{++} activated K^{+} channel that functions in the presynaptic membranes to negatively modulate neurotransmission (Wang, Saifee et al. 2001), and in *slo-1* mutants, I observed an interesting EPG (electropharyngeogram) phenotype.

The EPG is an extracellular recording of pharyngeal muscle currents during pumping (Raizen and Avery 1994; Avery, Raizen et al. 1995) – note that peristalses do not generate EPG currents. MC stimulation of pharyngeal muscles causes small positive spikes on EPGs, which either: 1) remain as single positive spikes if the MC stimulations fail 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, Lee et al. 1995) – see schematic in Fig. 14A. Further, the M3 neurons sometimes act during pumping to inhibit action potentials, which appear as small trains of negative EPG spikes (Raizen and Avery 1994) – also see schematic in Fig. 14A. Altogether, MC and M3 are the only neurons

whose functions are reflected in EPG recordings, and if MC and M3 are inactivated either by laser ablations or by the *eat-2* (Raizen, Lee et al. 1995; McKay, Raizen et al. 2004) and *eat-4* (Lee, Sawin et al. 1999) mutations, their corresponding spikes disappear from EPGs, including the single positive spikes that correspond to MC EPSPs (schematic in Fig. 14B, and an actual trace of *eat-2; eat-4* mutants is in Fig. 16B).

In *slo-1* mutants, however, Leon Avery and I found that even when MC and M3 functions were removed, the EPGs still contained EPSPs (quantified in Fig. 15A, and representative trace of *eat-2; eat-4; slo-1* EPGs are shown in Fig. 16C). This suggested the activation of a non-MC neuron to excite pharyngeal muscles by the *slo-1* mutation. Using laser ablations, I then found that killing M4 eliminated most of the novel EPSPs in *eat-2; eat-4; slo-1* animals (Fig. 15B), indicating that M4 was the neuron activated by *slo-1* to generate MC-independent EPSPs. Further, I found that *eat-2; eat-4; slo-1* animals also pumped faster than *eat-2; eat-4* animals (Fig. 17A), suggesting that activated M4 led to increased pumping as well.

Since M4 innervated the posterior isthmus and terminal bulb, the novel EPG EPSPs and increased pumping suggested specific activation of M4-TB synapses (recall that the posterior isthmus is involved in peristalsis, not pumping). However, since EPG signals and increased pumping could technically represent novel stimulation to the corpus and not the terminal bulb, I wished to test for specific activation of the terminal bulb. Thus, I examined the effects of *slo-1* on TB pumping in *eat-5* mutants, in which the TB pumps independently of the corpus, due to gap junction defects (Starich, Lee et al. 1996). Indeed, *eat-5; slo-1* animals had increased TB pumping compared to *eat-5* animals (Fig. 17B).

Based on the novel EPG EPSPs and increased TB pumping, it appeared that M4-TB synapses were activated in *slo-1* mutants. Since *slo-1* encodes a negative modulator of synaptic activity (Wang, Saifee et al. 2001), my results indicate that M4-TB stimulations are absent in *C. elegans* because M4-TB synapses are functionally silenced by a reduction in neurotransmission, i.e. their volumes were turned down.

M4-TB synapses in *C. elegans* were cholinergic

Other than *eat-2*, another gene that is required for MC to stimulate pharyngeal pumping is *eat-18* (Raizen, Lee et al. 1995; McKay, Raizen et al. 2004). 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, Raizen et al. 2004). Interestingly, in contrast to *eat-2; eat-4; slo-1* animals, I found that *eat-18; eat-4; slo-1* animals lacked EPSPs (Figure 18A). Thus, novel M4 activity in *slo-1* mutants required *eat-18* function, indicating that M4-TB synapses are cholinergic and use nicotinic channels. I then tested whether the nicotinic antagonist d-tubocurarine (Raizen, Lee et al. 1995) can also affect M4 EPSPs, and indeed, 100μM d-tubocurarine drastically reduced the number of EPSPs in *eat-2; eat-4; slo-1* EPGs (Figure 18B).

Discussion

The phenotype of *slo-1* mutants indicated that M4-TB synapses in *C. elegans* were functionally inactivated in evolution

To understand why M4 specifically does not stimulate the terminal bulb in the Rhabditidae family, I examined the relationship between M4 and the TB in detail in *C. elegans*. Using a *Pceh-28::snb-1::gfp* transgene, I found that M4-TB synapses are actually present in *C. elegans*. Further, in loss of *slo-1* mutants, I found evidence for M4-TB stimulation. Since *slo-1* encodes a presynaptic negative modulator of neurotransmission (Wang, Saifee et al. 2001), these findings indicated that the lack of M4-TB stimulation is due to changes in synaptic activity of the M4-TB synapses. That is, even though these synapses have the potential for function, the levels of neurotransmission were reduced to functionally irrelevant levels, unless a mutation like *slo-1* is present to increase neurotransmission. Thus, changes in synaptic strength appeared to be one significant way in which M4 function evolved in free-living soil nematodes.

Further, using the *eat-18* mutation and the drug d-tubocurarine, I showed that M4-TB synapses are cholinergic. M4 was previously reported as a cholinergic neuron based on staining for acetyltransferase (Rand and Nonet 1997), and the terminal bulb muscles had been shown to express surface nicotinic channels (McKay, Raizen et al. 2004). Yet, the functional relevance of these findings were unclear. My results here suggest that they might be the evolutionary remnants of previously active M4-TB synapses that became silenced in the Rhabditidae.

Since the effects of the SLO-1 channel on presynaptic membrane potential do not have to be static over time, and could possibly be quite dynamic, the observation of M4-TB simulation in *slo-1* mutants also raises the following question: were M4-TB synapses generally inactivated in *C. elegans* (and the Rhabditidae), or can M4-TB stimulations

actually occur under some specific conditions/assays? Our current results can not distinguish between the two scenarios, and I think that both are formally possible. However, since M4-TB stimulations are apparently absent (for wild type animals) in all laboratory conditions/assays that are known, I personally favor the presumption that M4-TB synapses are generally inactivated in wild type animals.

The genetic changes responsible for silencing M4-TB synapses

Since loss of *slo-1* led to M4-TB stimulations, can I conclude that silencing of M4-TB synapses in the Rhabditidae is due specifically to differences in the *slo-1* gene? Definitely not. I do think, however, that *slo-1* is an interesting candidate to investigate further for potentially relevant genetic differences. For example, differential expression of alternatively spliced BK channel isoforms can be of physiological importance (examples include Rosenblatt, Sun et al. 1997; Atkinson, Brenner et al. 2000), and an interesting hypothesis might be that the differential expression of a specific SLO-1 isoform in the Rhabditidae M4 is critical for inactivating M4-TB synapses.

On the other hand, Mark Steciuk had screened for additional mutations that can activate M4-TB stimulations. Interestingly, Mark identified several genes that also encode subunits of presynaptic ion channels that modulate synaptic transmission (personal communication), suggesting that genes that encode modulators of neurotransmission may be of general relevance. Yet, for any candidate gene, including *slo-1*, more direct evidence would be needed before one can conclude that gene's significance in the evolution of M4-TB

synaptic activity (e.g. specific sequence differences between each nematode family that correlate with the activity/inactivity of M4-TB synapses).

Materials and Methods

Strains

I used the wild type *C. elegans* strain N2, as well as mutant *C. elegans* strains that contained the following alleles: *slo-1(js379)* (Wang, Saifee et al. 2001), *unc-104(e1265)* (Hall and Hedgecock 1991; Otsuka, Jeyaprakash et al. 1991), *eat-2(ad465)* (McKay, Raizen et al. 2004), *eat-4(ky5)* (Lee, Sawin et al. 1999), *eat-5(ad1402)* – a putatively null deletion (835-2274bp of F13G3.8) that removes exons 2 thru 4, and *eat-18(ad1110)* (McKay, Raizen et al. 2004) . All strains were maintained at 19°C on NGMSR plates (Davis, Somerville et al. 1995) seeded with *E. coli* HB101 (Boyer and Roulland-Dussoix 1969).

Analysis of M4 synapses using the *Pceh-28p::snb-1::gfp* transgene

Pceh-28p::snb-1::gfp was generated by overlap extension PCR (Hobert 2002). I first amplified *Pceh-28* (2.4 kb sequence immediately upstream of the *ceh-28* start ATG) from genomic DNA, and *snb-1::gfp* from pSB120.65 (courtesy of Michael Nonet). I then fused the two together by additional PCR. The resulting PCR products were injected for transformation either into wild type N2 animals or *unc-104(e1265)* animals. Coinjection markers used were either pRAK3 [rol-6(d)] (Davis, Somerville et al. 1995) or a *Podc-1::gfp* construct which expresses in intestinal cells (unpublished observations). *Podc-1::gfp* was generated by overlap extension PCR between *Podc-1* (3.0kb sequence upstream of the start

ATG of *odc-1* (Macrae, Plasterk et al. 1995)) amplified from genomic DNA, and *gfp*, amplified from pPD95.75 (Fire lab vector kit). 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)

Electropharyngeograms (EPGs) in *slo-1* mutants

Electropharyngeograms on exposed pharynxes bathed in Dent's saline and 1μM serotonin (5HT) were performed as previously described (Davis, Somerville et al. 1995), with one exception. Although MC-independent EPSPs in *slo-1* mutants were present in EPGs performed using standard Dent's saline ($[Ca^{++}]_{ext}$ =3.0mM), those EPSPs were larger in amplitude and easier to distinguish when I increased $[Ca^{++}]_{ext}$ to 5.0mM. Therefore, all results in Fig. 15 and 16 were derived from EPGs using modified Dent's containing 5.0mM $[Ca^{++}]_{ext}$, although the results were qualitatively similar when using standard Dent's saline. EPG currents were digitally sampled and recorded as previously described (Davis, Somerville et al. 1995). Digitized EPG recordings were then viewed and analyzed using Igor Pro 4.05 from Wave Metrics (Lake Oswego, OR, USA). The quantification of EPSP rates on EPGs were assisted by Igor Pro procedures (see below) that scanned for single positive spikes that do not precede full pharyngeal action potentials, which were designated as EPSPs.

Laser ablations

MC and M3 laser ablations in wild type and *slo-1* animals (and the subsequent EPGs) were done by Leon Avery following 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 all operated animals were subsequently grown on HB101 seeded plates containing 5mM arecoline. 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 by 5mM arecoline (Leon Avery, personal communication). The EPGs of mock ablated *eat-2; eat-4; slo-1* animals grown on 5mM arecoline were similar to EPGs of animals grown under standard conditions, indicating that growth on 5mM arecoline had no major effects on the EPGs.

Analysis of EPSPs in EPGs of *slo-1* mutants

I developed procedures in the Igor Pro environment to quantify the rate of single positive spikes seen on EPGs. The underlying idea was to determine action potentials in EPG traces by identifying E2 spikes and R1 spikes (Raizen and Avery 1994), since they are the largest spikes in the action potential and are not affected by the laser operations or genetic mutations used in our experiments. Positive spikes not determined to be a part of an action potential were then designated single positive spikes (i.e. EPSPs).

First, all positive and negative spikes were identified in the following manner. EPGs were high pass filtered (>1Hz) using a Fast Fourier Transform based algorithm to remove baseline drift. Then, threshold parameters, t_E and t_R, were calculated as 20% of the E2 and R1 spike amplitudes of the smallest identifiable action potential, which allowed

normalization to variable spike amplitudes caused by *slo-1* effects (data not shown) and inherent size differences of mutant animals. Positive peaks were then selected as local maxima meeting the following criteria: 1) Peak amplitude greater than t_E (global thresholding), and 2) Peak amplitude greater by t_E compared to a local baseline calculated as the average amplitude 10-25ms before and after the putative peak (local thresholding). Negative peaks were identified by applying identical thresholding steps to local minima in the EPG, but using the parameter t_R.

Then, to identify E2 and R1 spikes, all positive spikes (and similarly negative spikes) that were within 20 ms of each other were grouped together and replaced by the largest positive (or negative) amplitude spike within each group. Amongst these spikes, a positive spike followed within 500ms by a negative spike, with no other intervening spikes, were designated E2 and R1 spikes of an action potential. All other positive spikes were then designated as single positive spikes, i.e. EPSPs.

Pump rate assays

I measured *eat-2; eat-4* and *eat-2; eat-4; slo-1* pumping rates by selecting gravid adults and counting the number of pumps in one minute under a dissecting microscope at 18°C. For *eat-5* and *eat-5; slo-1* animals, I instead counted TB pumping rates in L1 larvae within 6 hours of hatching (rather than adults) because the uncoupled terminal bulb phenotype in *eat-5* mutants is virtually 100% penetrant in young larvae, but significantly less so in older larvae and adults (Starich, Lee et al. 1996). I measured *eat-5* and *eat-5; slo-1* TB

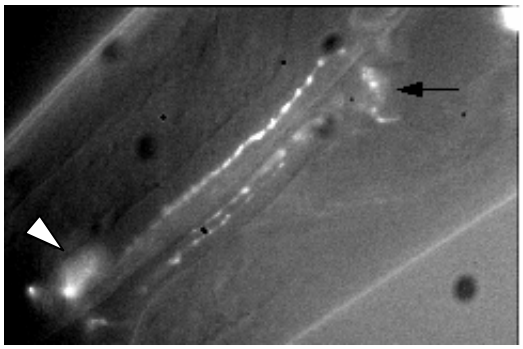
pump rates by counting for one minute at 18°C under a Zeiss Axiophot microscope using a 20x objective.

d-tubocurarine studies

For the 100μM d-tubocurarine studies, EPGs were recorded for 2 minutes in 100μl of 5.0mM $[Ca^{++}]_{ext}$ Dent's + 5HT, followed by the addition of another 100μl of either the identical saline (control) or the same saline with 200μM d-tubocurarine added (experiment), to achieve a final concentration of 100μM d-tubocurarine. The rate of EPSPs (i.e. single positive spikes) was analyzed as described above.

Figure 13 – M4 innervation of the terminal bulb revealed by *Pceh-28::snb-1::gfp*

A



B

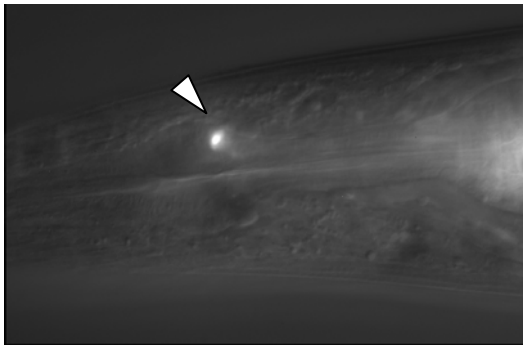


Figure 13 – M4 innervation of the terminal bulb revealed by *Pceh-28::snb-1::gfp*

A) In *Pceh-28::snb-1::gfp* transgenic *C. elegans* animals, the *Pceh-28* promoter drives M4 specific expression of SNB-1::GFP, a synaptic vesicle marker. In these animals, punctate expression of SNB-1::GFP along M4 axons seen in the isthmus and terminal bulb (TB), indicating M4 innervation of the isthmus and TB. White arrowhead points to the M4 cell body. Black arrow points to puncta in the TB.

B) To confirm that the SNB-1::GFP expression in *Pceh-28::snb-1::gfp* corresponded truly to synaptic structures, *Pceh-28::snb-1::gfp* was expressed in *unc-104* mutants, which should cause mislocalization of normally synaptic proteins to the cell body. Indeed, SNB-1::GFP in *unc-104* animals harboring the *Pceh-28::snb-1::gfp* transgene were all mislocalized to the M4 cell body, indicated by the white arrowhead.

Figure 14 – Schematics of EPGs (electropharyngeograms) in *C. elegans*

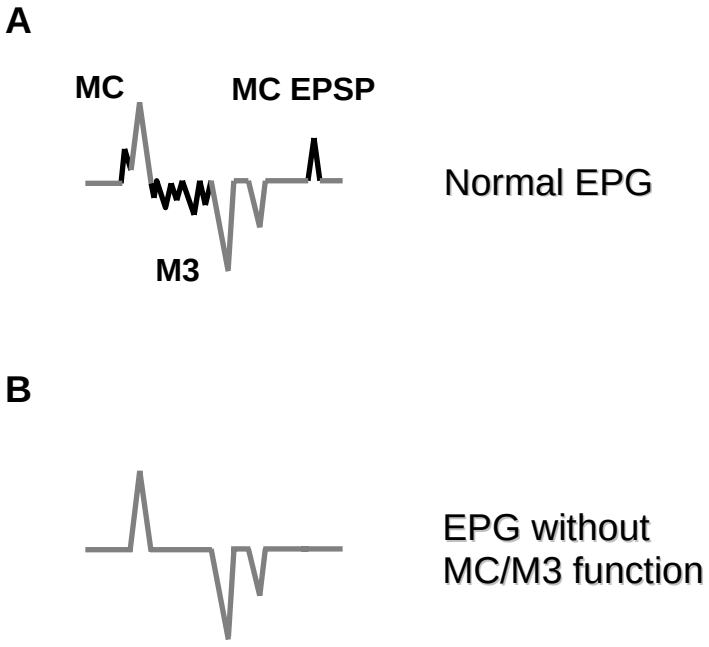


Figure 14 – Schematics of EPGs (electropharyngeograms) in *C. elegans*

A) A schematic of *C. elegans* EPGs (electropharyngeograms), a current recording from the corpus and terminal bulb 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 were 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 MC EPSP on the right. The neurogenic spikes (i.e. due to MC and M3) are drawn in black, whereas myogenic currents are drawn in gray.

B) If MC and M3 are inactivated, either by laser ablation or mutations such as *eat-2* and *eat-4*, then the resulting EPGs contain only myogenic currents. In particular, there should be no single positive spikes, i.e. MC EPSPs.

Figure 15 – Loss of *slo-1* activated M4 to generate EPSPs on EPGs

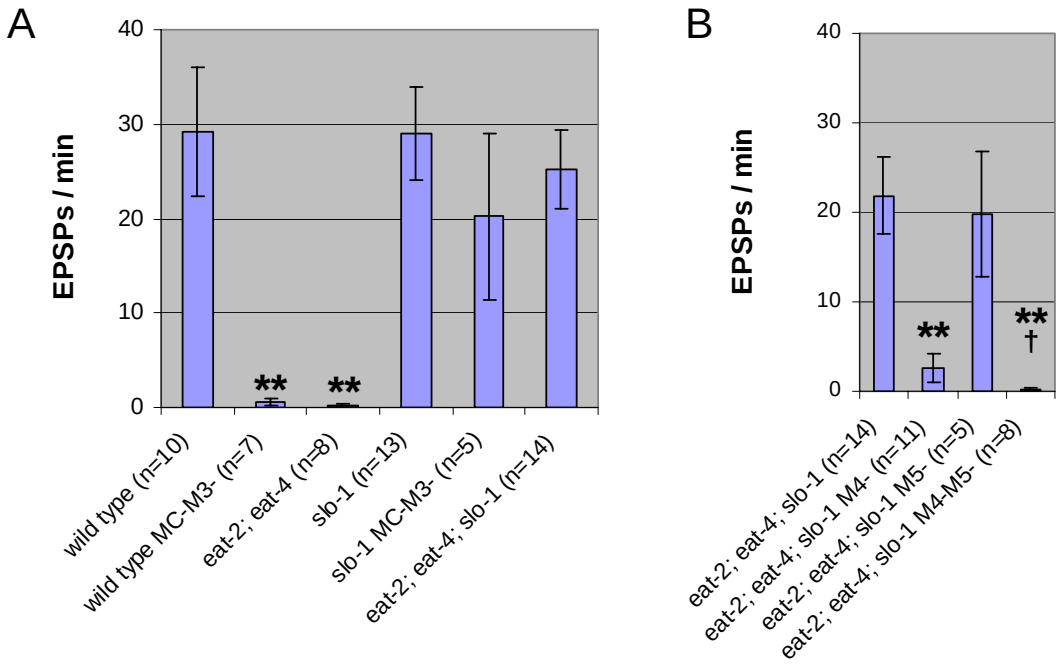


Figure 15 – Loss of *slo-1* activated M4 to generate EPSPs on EPGs

A) In the wild type background, loss of MC and M3 function eliminates all neurogenic EPG signals, including EPSPs. In the *slo-1* background, EPSPs were present even when MC and M3 functions were removed. Shown in the graph is quantification of EPSP rates 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. Asterisks indicate significant differences from wild type by two-tailed T test (** is $P<0.01$).

B) Laser ablation of M4 drastically reduced EPSPs in *eat-2; eat-4; slo-1* animals, whereas ablation of M5, another neuron that innervates the *C. elegans* terminal bulb, did not. Killing both M4 and M5 eliminated all EPSPs. Thus, M4 was activated by the *slo-1* mutation to cause MC-independent EPSPs on EPGs, and M5 may be slightly activated as well. Asterisks indicates significant differences from control *eat-2; eat-4; slo-1* animals by two-tailed T test (** is $P<0.01$). Dagger indicates significant differences from M4- *eat-2; eat-4; slo-1* animals by two-tailed U-test († is $P<0.05$).

Figure 16 – EPG traces illustrating M4 EPSPs due to loss of *slo-1*

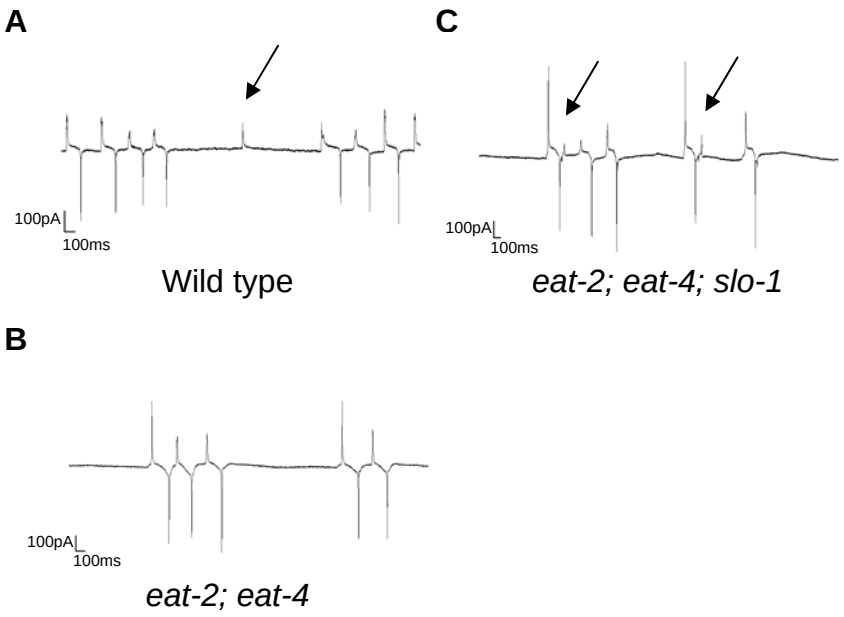


Figure 16 – EPG traces illustrating M4 EPSPs due to loss of *slo-1*

A) Wild type animals have MC EPSPs on EPGs.
B) *eat-2; eat-4* animals do not have EPSPs.
C) Loss of *slo-1* caused *eat-2; eat-4; slo-1* animals to have MC-independent EPSPs (due to M4, as shown in Figure 14 – also see text).

Arrows indicate EPSPs.

Figure 17 – Loss of *slo-1* caused increased TB pumping

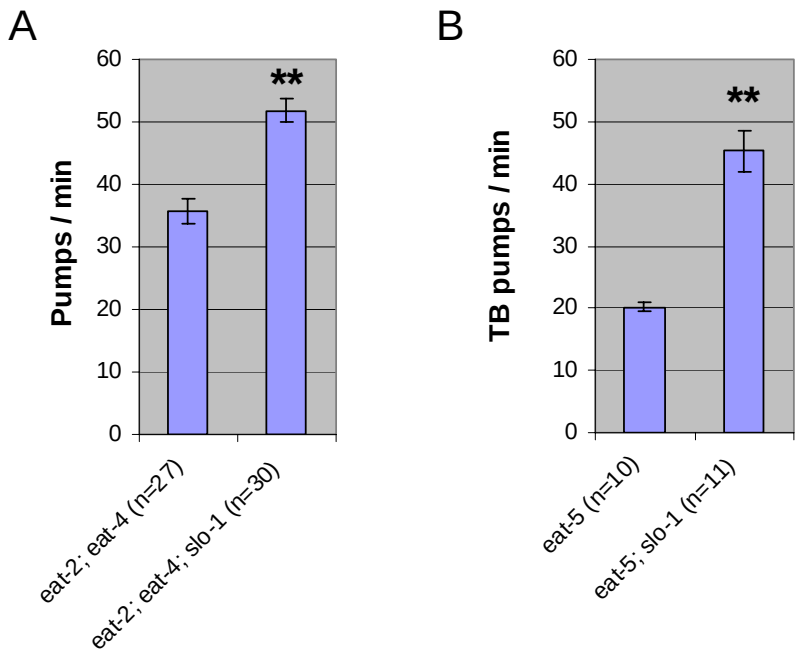


Figure 17 – Loss of *slo-1* caused increased TB pumping

A) *eat-2; eat-4; slo-1* animals have increased pumping compared to *eat-2; eat-4* animals
B) TB pumping is uncoupled from corpus pumping in *eat-5* animals. *eat-5; slo-1* animals have increased TB pumping compared to *eat-5* animals.

Asterisks indicate statistically significant differences by two-tailed T-test (** for $p<0.01$).

Figure 18 – M4-TB synapses are cholinergic

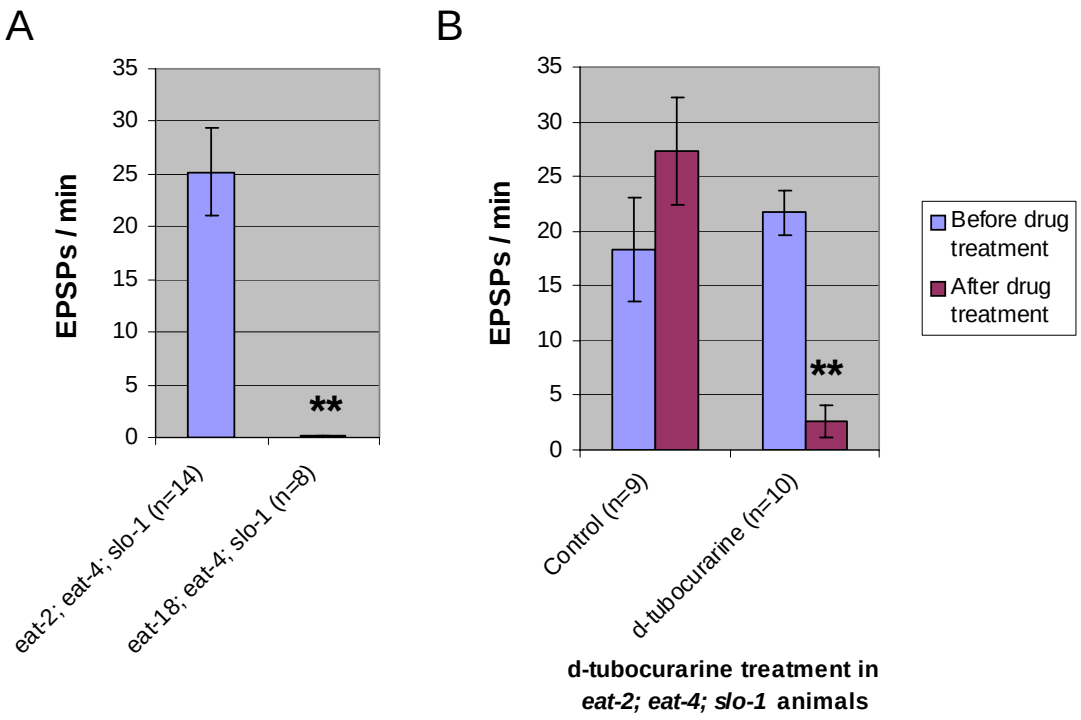


Figure 18 – M4-TB synapses are cholinergic

A) Although both *eat-2* and *eat-18* are required for MC function, *eat-2* specifically encodes a nicotinic channel subunit at the MC-corpus neuromuscular junction, whereas *eat-18* is required for the surface expression of all pharyngeal nicotinic channels, including in the terminal bulb. While *eat-2; eat-4; slo-1* animals have EPSPS (due to M4), *eat-18; eat-4; slo-1* animals do not have EPSPs on EPGs. The asterisk indicates statistically significant difference between *eat-18; eat-4; slo-1* and *eat-2; eat-4; slo-1* by two-tailed T test (** for $P<0.01$).

B) EPSP rates in *eat-2; eat-4; slo-1* animals where reduced after treatment with 100μM d-tubocurarine (dTC), a nicotinic channel antagonist. A control treatment was also performed, where empty saline without dTC was used, which did not reduce EPSPs. The asterisk indicates statistical significance between d-tubocurarine and control treatment, by two-tailed T test applied to the difference in EPSP rates (i.e. after treatment minus before treatment) in individual animals (** for $P<0.01$).

CHAPTER FIVE

Loss of *ceh-28* caused peristalsis defects in *C. elegans*

Introduction

Motivation for generating a deletion allele of the *ceh-28* gene

In Chapters 2 and 3, I showed that significant changes in peristalses occurred in the evolution of free-living soil nematodes, both with regard to where peristalses occurred and what pharyngeal neuron is responsible for stimulating peristalses. What might be the underlying mechanisms for these changes? Unfortunately, the molecular mechanisms that govern peristalsis and its regulation by M4 were entirely unknown even in *C. elegans*.

One gene, however, offered a clue. The gene *ceh-28* encodes a homeodomain transcription factor in *C. elegans* and was previously shown to express specifically in M4 (Brian Harfe, personal communication). Nonetheless, despite this suggestive expression pattern, the actual physiological function of *ceh-28* had not been determined. Therefore, I requested a knockout allele of *ceh-28* from the National Bioresource Project (a.k.a. the Japanese Knockout Consortium), to test if *ceh-28* mutants have phenotypes in peristalsis and M4 function.

Results

tm1258 is a probable null deletion allele of *ceh-28*

With luck, the NBRP generated a deletion allele in the *ceh-28* gene in very little time (~4 months). The allele was named *tm1258*, and consisted of a 972 bp deletion that began 107 bp upstream of the start ATG and ended in the third exon (Figure 19). Based on the location and extent of the region deleted, *tm1258* was a putatively null allele of *ceh-28*.

Defective peristalsis in *ceh-28* deletion mutants

ceh-28(tm1258) mutants were homozygous viable and had the classic Eat phenotype (Avery 1993), i.e. a generally starved appearance with pale, thin bodies, and reduced growth rates and brood sizes (qualitative observations – not quantified). When examined in detail, *ceh-28(tm1258)* animals showed defects in peristalsis, which explained the Eat phenotype, and also corresponded with the previously known *ceh-28* expression in M4.

Interestingly, the peristaltic defects were dependent on developmental stage, and progressively worsened as the animals grew. In larvae immediately after hatching, peristalsis actually appeared relatively normal. Yet, by ~15 hours after hatching, i.e. late L2 or early L3 stage, the corpus and anterior isthmus were typically filled with bacteria (Fig. 20), and peristaltic motions were either severely defective or entirely absent (see supplemental videos in Appendix B). The absolute extent to which peristalses were defective in *ceh-28(tm1258)* animals was unclear because the posterior isthmus muscles still showed some slight movements and some bacteria still passed through to the terminal bulb (see supplemental videos in Appendix B). It was not clear whether these motions corresponded to some remaining ability of the posterior isthmus muscles to conduct peristalses, or if those motions simply corresponded to the posterior isthmus being forced open by corpus and anterior isthmus pumping and shoving bacteria through.

Compared to the peristaltic defects in M4 ablated *C. elegans*, the *ceh-28* phenotype was certainly less severe. Whereas *ceh-28* was not absolutely required for peristalsis, especially in the earlier stages of larval development, M4 ablated animals have stuffed pharynxes and no visible peristalses even in the L1 developmental stage, and typically do not

develop past the L1 larval stage (Avery and Horvitz 1987). Further, these differences in phenotype between *ceh-28* mutants and M4 ablated animals were not specific to the *tm1258* allele, as Peter Okkema's laboratory independently generated another *ceh-28* deletion allele, named *cu11* (*cu11* removed most of the homeobox regions, i.e. ~3rd and 4th exons), and the phenotypes of *ceh-28(cu11)* animals were indistinguishable from *ceh-28(tm1258)* animals, i.e. also milder than M4 ablated animals (Peter Okkema, personal communication).

No obvious anatomical defects in M4 of *ceh-28* mutants

Homeobox genes are known to be important for proper anatomical development and cell differentiation – therefore, I looked for such defects in the M4 neuron in *ceh-28* mutants. By DIC (differential interference contrast) microscopy the M4 nucleus in *ceh-28* mutants had normal morphology and position within the pharynx (Fig. 21A). Also, I found that M4 in *ceh-28(tm1258)* mutants still properly expressed *Pceh-28::gfp*; and when I examined the expression patterns of *Pceh-28::gfp* and *Pceh-28::snb-1::gfp* in *ceh-28(tm1258)* mutants, they revealed that M4 had normal axonal and synaptic morphology (see Fig. 21B). Taken together, these results suggested that M4 development, specification, and ultrastructure were largely normal, and the M4 defects caused by loss of *ceh-28* are probably more functional or physiological in nature.

Effects on M4-TB stimulation in *ceh-28* mutants

In Chapter 4, I found that in addition to M4's normal function in stimulating peristalses, M4 can also stimulate terminal bulb pumping in mutant backgrounds such as *slo-*

1. Therefore, I wanted to test if loss of *ceh-28* affected that aspect of M4 function as well. In other words, is *ceh-28* important specifically for M4 to stimulate peristalsis, or does *ceh-28* affect all of M4's functions?

To address this, I tested whether *ceh-28* is required for the *unc-36* mutation to increase TB pumping in the *eat-5* background. Like *slo-1*, *unc-36* is a modulator of neurotransmission (Schafer, Sanchez et al. 1996), and loss of *unc-36* can similarly activate M4-TB stimulations (Mark Steciuk, personal communication). For this experiment, I used *unc-36* rather than *slo-1* because the genetic crosses required to generate *eat-5; unc-36; ceh-28* mutants were much easier than *eat-5; slo-1; ceh-28* mutants (the ease of strain constructions was critical since a high degree of larval lethality occurred in the *eat-5; ceh-28* background).

I found that while *eat-5; unc-36* animals had higher pumping rates than *eat-5* animals, as expected, *eat-5; unc-36; ceh-28* and *eat-5; ceh-28* animals had virtually identical TB pump rates (Fig. 22). Thus, when *ceh-28* is mutated, the ability for *unc-36* to increase TB pumping in the *eat-5* background was eliminated, suggesting that *ceh-28* affected both the peristalsis and the TB stimulation aspects of M4 function.

Possible expression of *ceh-28* in M2 in *C. elegans*

Interestingly, even though *ceh-28* was previously shown to express specifically in M4 using a *Pceh-28::gfp* transgenic construct (Brian Harfe, personal communication), Mark Steciuk found that a *Pceh-28::gfp* transgenic line he created showed M2 as well as M4 expression (Mark Steciuk, personal communication). Mark's *Pceh-28::gfp* construct

included only promoter sequences upstream from the *ceh-28* start ATG, whereas Brian Harfe's *Pceh-28::gfp* construct included the same upstream sequences, but also contained downstream coding sequences (308bp) down to the second exon (see schematic in Fig. 23A). To me, this suggested two things: 1) M2 in *C. elegans* has the potential to express *ceh-28*, and 2) this expression is normally prevented by a repressor sequence located between the start ATG and mid-2nd exon of *ceh-28*. Since M2, rather than M4, stimulated peristalsis specifically in the Panagrolaimidae family, these implications suggested potentially interesting links between the two neurons.

However, since Mark Steciuk and Brian Harfe's *Pceh-28::gfp* results came from separate experiments, I wanted to confirm their results by expressing the two *Pceh-28::gfp* constructs of differing lengths in a head-to-head experiment. Intriguingly, both forms of *Pceh-28::gfp* in my experiment showed M2, as well as M4, expression (Fig. 23B). Thus, although my results confirmed the potential for *ceh-28* expression in M2, my results also argued against the presence of a repressor in the suspected genomic region. Adding further mystery, preliminary experiments in Peter Okkema's lab showed that their version of *Pceh-28::gfp* (the exact sequence of the *Pceh-28* promoter used was not communicated) had specific M4 expression in wild type animals, but possible M2 expression as well in *ceh-28(cu11)* mutants (Peter Okkema, personal communication).

The firm conclusion that can be drawn from these expression studies is that M2 in *C. elegans* has some potential to express *ceh-28*. What is unclear is whether M2 expression of *ceh-28* occurs normally, or is dependent on specific perturbations/conditions. Nonetheless, this potential for M2 to express *ceh-28*, like M4, suggests an interesting molecular/genetic

link between these two neurons and their differing abilities to stimulate peristalses in different families of free-living soil nematodes.

Discussion

ceh-28 is important for peristalsis and M4 function in *C. elegans*

To better understand the mechanisms that underlie peristalsis and M4 function in *C. elegans*, I examined the effects of knocking out the gene *ceh-28*, which had previously been shown to express specifically in M4 (Brian Harfe, personal communication). In *ceh-28* mutants, I found obvious defects in peristalsis, indicating that *ceh-28* is an important gene for proper stimulation of peristalsis by M4. Since the nuclear, axonal, and synaptic morphology of M4 in *ceh-28* mutants were all relatively normal and M4 also retained the ability to express *ceh-28*, loss of *ceh-28* probably caused functional defects in M4, rather than defects in M4 development, anatomy, or neuronal specification. Interestingly, a recent report showed that mutating *ceh-2*, another homeobox gene, also caused apparently functional (i.e. not developmental) defects in the M3 pharyngeal neurons (Aspock, Ruvkun et al. 2003).

Compared to M4 ablated animals, however, the defects in *ceh-28* mutants were milder, especially in newly hatched larvae. Thus, *ceh-28* must not be absolutely required for peristalsis. To identify other genes and mechanisms that are important for peristalsis, the *ceh-28* mutant can be used in enhancer or suppressor screens. Some potentially interesting types of genes that might be isolated in such screens include: 1) other transcription factors, since homeodomain transcription factor often work in tandem with other transcription factors, and 2) downstream targets genes whose expression is controlled by CEH-28.

Further, genes that function either in the neuron M4 or the downstream posterior isthmus muscle can be isolated in such screens. Altogether, in addition to demonstrating the significance of *ceh-28* in peristalsis and M4 function, the *ceh-28* mutant might also serve as a useful tool to find additional relevant genes/molecules in *C. elegans*.

Possible evolutionary implications of *ceh-28* expression in M2

The *Pceh-28::gfp* experiments showed that M2 in *C. elegans* has the potential for expressing *ceh-28,* which indicated that M2 and M4 are similar in some way at the molecular and genetic levels. Thus, the two neurons might use similar mechanisms to stimulate peristalsis in their respective families (i.e. M2 in the Panagrolaimidae, and M4 in the other three families). If this is true, it will be interesting to determine how these mechanisms are differentially expressed and active in M2 and M4, in their respective families.

One potential hypothesis is that *ceh-28* itself is differentially expressed, i.e. M2 in the Panagrolaimidae expresses *ceh-28* and M4 in the other three families express *ceh-28,* and that this differential expression is important for determining which neuron stimulates peristalses. Given the current results, this hypothesis is relatively speculative, although it would have been strengthened if the *Pceh-28::gfp* experiments clearly demonstrated a repressor element responsible for preventing *ceh-28* expression in the *C. elegans* M2. Unfortunately, my results did not support the presence of such a repressor. Nonetheless, since the exact pattern of *ceh-28* expression in *C. elegans* is still not clear, and the expression pattern of *ceh-28* in other nematodes species is also undetermined, it still might be that *ceh-28* is differentially expressed in the different nematode families.

To clarify and further investigate these issues, I am currently raising antibodies to CEH-28. First, I hope to use these antibodies to determine whether M2 in *C. elegans* normally does express *ceh-28*. Second, if the antibodies also cross react with the homologues of CEH-28 in other nematode species, then I would like to directly test the hypothesis that *ceh-28* is differentially expressed in M2 in the Panagrolaimidae and M4 in the other three families.

Materials and Methods

Strains

ceh-28(tm1258) was generated by the National Bioresources Project (Japan). After I received the *ceh-28(tm1258)* mutants from the NBRP, I outcrossed the allele 5x before analyzing the mutant phenotypes. During outcrossing I tracked the *tm1258* deletion by the starved/pale phenotype, and then I confirmed the presence of the deletion by PCR after outcrossing. Other alleles used during the analysis of *ceh-28(tm1258)* include *eat-5(ad1402)*, and *unc-36(e251)* (Schafer, Sanchez et al. 1996). Strains were maintained at 19°C on NGMSR plates (Davis et al., 1995) seeded with *E. coli* HB101 (Boyer and Roulland-Dussoix, 1969).

Visual observation of peristaltic defects in *ceh-28* mutants

Standard egg preps by hypochlorite treatment (Lewis and Fleming 1995) were used on wild type and *ceh-28(tm1258)* animals, and the resulting eggs were place in M9 solution overnight to allow hatching and developmental synchronization of the resulting larvae. On

the next day, the synchronized larvae were then plated on HB101 plates and observed at 5 hour intervals – i.e. immediately after plating, 5 hours post, 10 hours post, etc.

To observe peristalses, the larvae were coverslipped on NGMSR pads in 3µl of 10mM 5HT in M9 with HB101 (at a density of ~1 plate/100-200µl). I used 5HT in order to slow down the animals, to permit detailed observation of posterior isthmus peristalses. I used bacteria in order to stimulate peristalsis (even in wild type larvae, peristalses tended to be infrequent if placed in 10mM 5HT M9 without any bacteria – see Chapter 2). Peristaltic motions were then observed using a Zeiss Axiophot microscope using 63x and 100x objectives. Videos were taken with a Hitachi KP-160 CCD camera and digitized using Adobe Premiere v6.5. Compression of videos into MPEG files were done via SmartVideoConverter v1.5.15 (DoEase Software).

Analysis of M4 structure in *ceh-28(tm1258)*

I used DIC microscopy to observe M4 nuclei in *ceh-28(tm1258)* mutants. I also crossed *Pceh-28::gfp* and *Pceh-28::snb-1::gfp* into *ceh-28(tm1258)* mutants from strains PD4625 and DA1833, in order to observe M4 axonal and synaptic morphology. Fluorescence imaging on these transgenic animals was performed as described in Chapter 4.

TB pumping rate assay

In *eat-5*, *eat-5; unc-36*, *eat-5; ceh-28*, and *eat-5; unc-36; ceh-28* animals, I counted TB pumping rates in L1 larvae within 4 hours of hatching. I measured TB pump rates by

counting for one minute at 18°C under a Zeiss Axiophot microscope using a 40x long-distance objective.

Analysis of *Pceh-28::gfp* expression patterns

Pceh-28::gfp containing two different lengths of *Pceh-28* were amplified using overlap extension PCR (Hobert 2002) . The shorter construct contained only 2.4kb upstream sequence (from the *ceh-28* start ATG), and corresponded to the *Pceh-28::gfp* previously used by Mark Steciuk. The longer construct contained an additional 308bp down to the middle of the second *ceh-28* exon, and corresponded to the *Pceh-28::gfp* previously used by Brian Harfe. Both were coinjected into wild type N2 animals using *Podc-1::gfp* as the coinjection marker, as described above. Fluorescence imaging of the ensuing expression patterns were also performed as described above.

Figure 19 – Schematic of the *tm1258* deletion

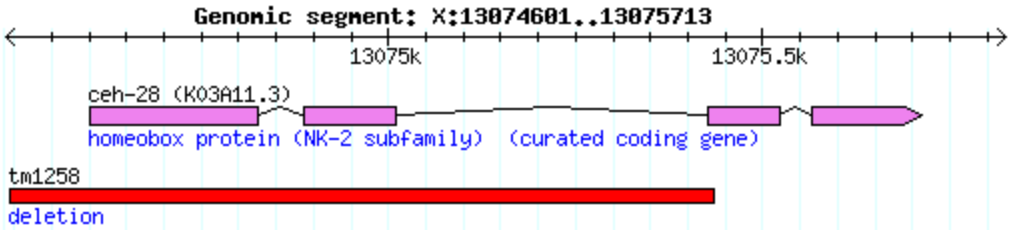


Figure 19 – Schematic of the *tm1258* deletion

The *tm1258* allele has a deletion in the *ceh-28* gene that begins 107 bp upstream of the start ATG and ends in the third exon. Based on the location of this deletion, *tm1258* is a putatively null allele of *ceh-28*.

(Figure obtained from www.wormbase.org)

Figure 20 – Stuffed pharynx phenotype in *ceh-28(tm1258)* mutants

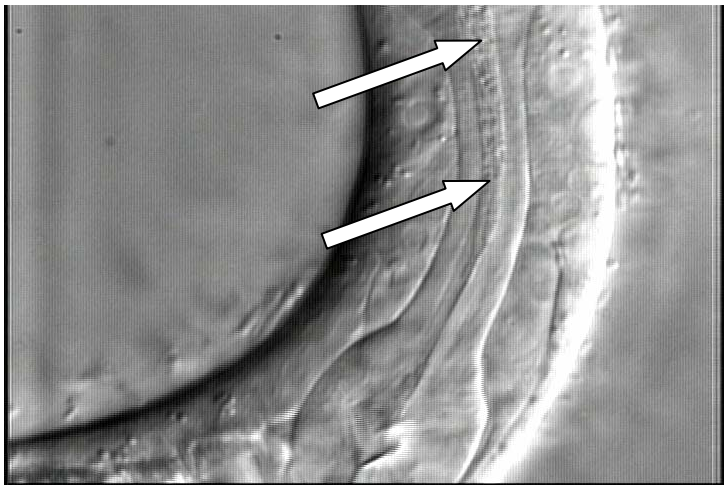
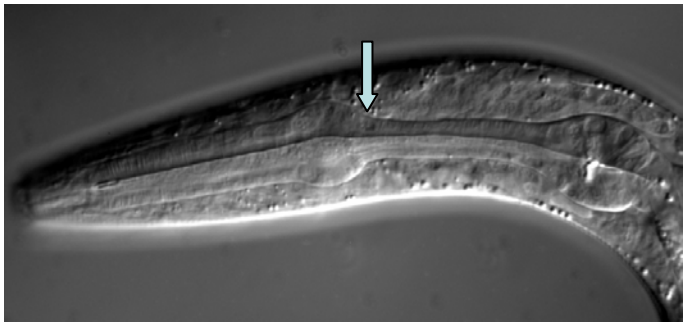


Figure 20 – Stuffed pharynx phenotype in *ceh-28(tm1258)* mutants

Defective posterior isthmus peristalsis in *ceh-28(tm1258)* mutants caused the corpus and anterior isthmus to become filled with bacteria. In this picture, the white arrows point to the filled lumen at the very posterior end of the corpus and in the anterior isthmus. This picture was taken from a *ceh-28(tm1258)* larva 20 hours after hatching. Also see videos of defective peristalsis motions in *ceh-28(tm1258)* animals (listed in Appendix B).

Figure 21 – Normal M4 morphology and structure in *ceh-28(tm1258)* mutants

A



B

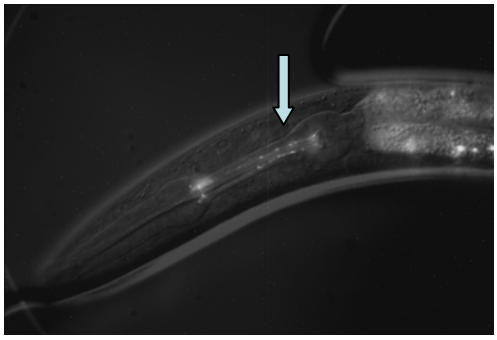


Figure 21 – Normal M4 morphology and structure in *ceh-28(tm1258)* mutants

A) M4 nucleus (white arrow) has normal shape and size in *ceh-28(tm1258)* mutants, as seen with DIC (differential interference contrast) light microscopy.

B) *Pceh-28::snb-1::gfp* expression in *ceh-28(tm1258)* mutants show that M4 axonal trajectory and synaptic locations are largely similar to those of wild type – i.e. they pass through and synapse onto the isthmus and terminal bulb. White arrow indicates punctate SNB-1::GFP expression that corresponds to M4 synapses.

Figure 22 – Loss of *ceh-28* prevented *unc-36* from activating TB pumping in the *eat-5* background

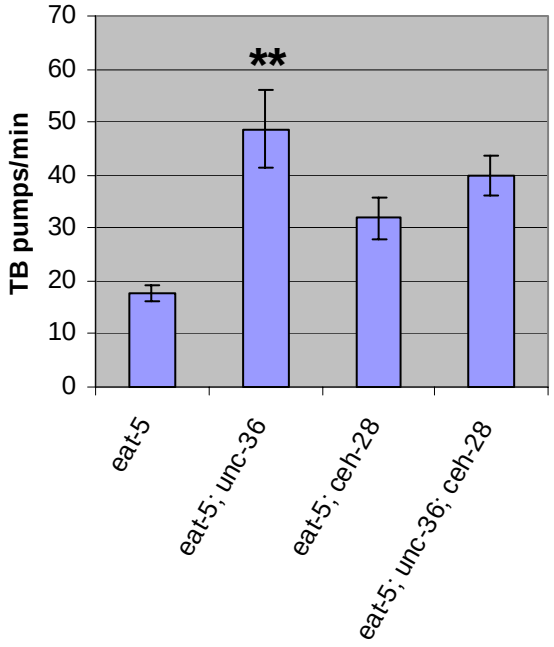


Figure 22 – Loss of *ceh-28* prevented *unc-36* from activating TB pumping in the *eat-5* background

Normally, *unc-36* can increase TB pumping in the *eat-5* background by activating M4 (Mark Steciuk, personal communication). Loss of *ceh-28*, however, prevented *unc-36* from increasing TB pumping. Asterisk indicates significant difference between *eat-5* and *eat-5; unc-36* by two-tailed T-test (** for $P<0.01$).

Figure 23 – *Pceh-28::gfp* showed potential for M2 expression in *C. elegans*

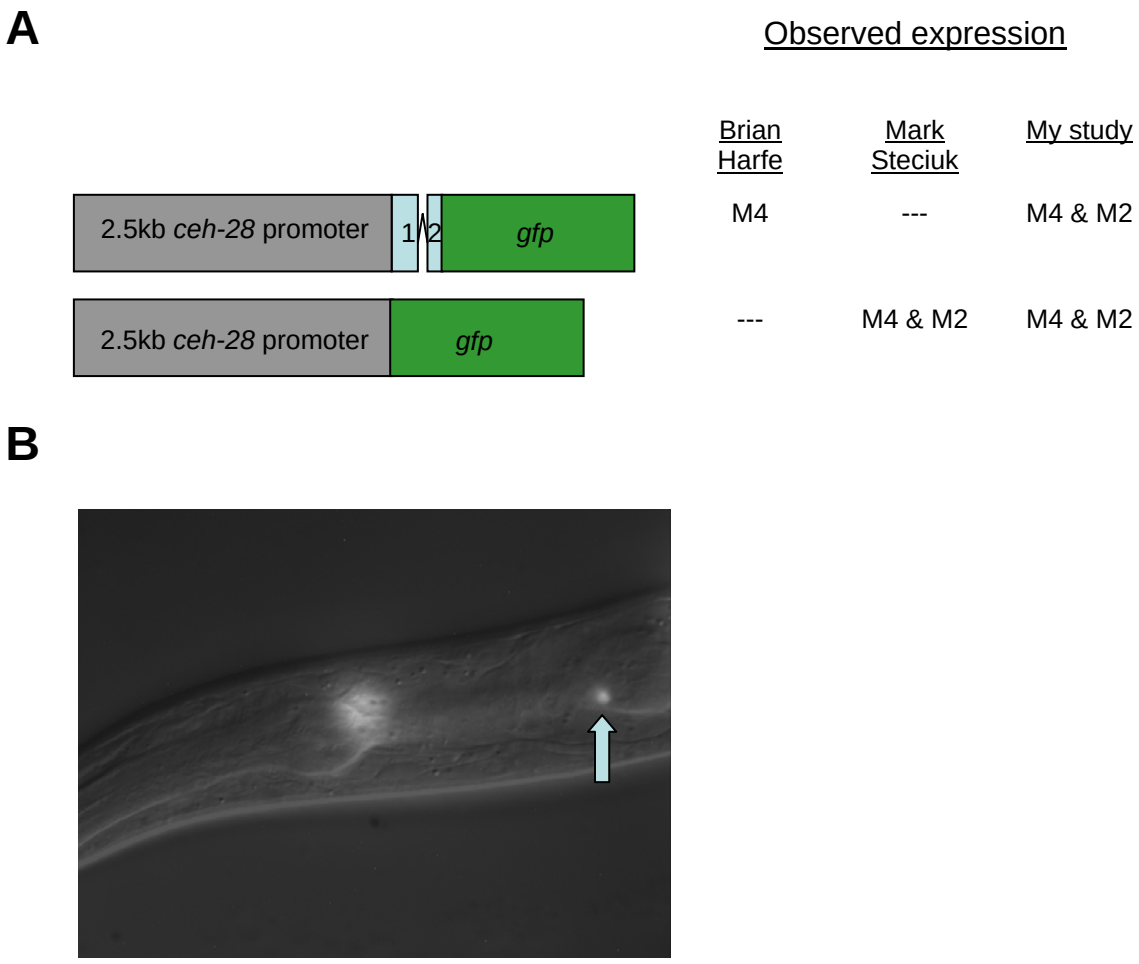


Figure 23 – *Pceh-28::gfp* showed potential for M2 expression in *C. elegans*

A) Previously, a *Pceh-28::gfp* construct used by Brian Harfe, containing ~2.5kb sequence upstream of the start ATG, as well as ~0.3kb downstream sequence into the second exon, showed M4 specific expression. However, a *Pceh-28::gfp* construct used by Mark Steciuk, containing only the ~2.5kb sequence upstream of the start ATG, showed M4 as well as M2 expression. I personally tested *Pceh-28::gfp* constructs containing the same sequences Brian Harfe and Mark Steciuk used, in a head-to-head experiment, and found that both constructs showed expression in M4 and M2 in my study.

B) A representative photo of the M2 expression observed in *Pceh-28::gfp* transgenic animals. The white arrow points out the expression in M2. Note that M4 expression (out of focus in a different plane) is also seen in the corpus.

CHAPTER SIX

General discussion

Overall summary

In this study, I characterized the behavioral, neuronal, and potential genetic differences in pharyngeal behaviors of free-living soil nematodes. Video microscopy demonstrated distinct differences in isthmus and terminal bulb behaviors. Laser ablations demonstrated that neuronal regulation of these behaviors involved broad changes in M4 downstream functions, as well as a specific change in M2 function. *slo-1* studies suggested that M4-TB synapses are functionally silenced in *C. elegans* and the Rhabditidae family. *ceh-28* studies showed that *ceh-28* is important for peristalses, and suggested that M2 and M4 may be linked molecularly/genetically, such as by the differential expression of *ceh-28*.

Altogether, these results suggested that the following neuronal and genetic mechanisms may contribute to the evolution of pharyngeal feeding behaviors: 1) co-option at the neuronal level (as exemplified by M4), 2) changes in the strengths of synaptic connections (as exemplified by the activation of M4-TB synapses in *slo-1* mutants), and 3) changes in the expression of transcription factors (as exemplified by the hypothesis concerning differential *ceh-28* expression). The last proposal is much more speculative, and would require additional molecular/genetic studies in non-*C. elegans* species. The first and second ideas would also be enriched by additional molecular/genetic studies, especially in non-*C. elegans* species, to identify the specific underlying genetic differences that evolved.

The use of nematodes as a model to study the evolution of behaviors

In many ways, the free-living soil nematode species that I examined were quite amenable to comparative studies. Like *C. elegans*, their pharyngeal behaviors also occurred

in simple, stereotyped patterns, permitting easy characterization and comparisons to *C. elegans* and each other. Protocols for laser ablations were also adapted to other nematodes with relative ease, which allowed the functions of single, homologous neurons to be characterized and compared. Finally, the wealth of techniques in *C. elegans* allowed relatively rapid generation of hypotheses concerning the molecular/genetic mechanisms by which the behavioral and neuronal differences evolved.

The main difficulty, however, concerned the lack of genetic/molecular techniques in non-*C. elegans* species. While the use of *C. elegans* allowed rapid generation of hypotheses concerning the important molecular and genetic changes that evolved, conclusive study of those changes would require genetic/molecular techniques in non-*C. elegans* species. Yet, adapting *C. elegans* techniques to other nematodes was not always straightforward. For example, other members of the laboratory and I have failed in creating transgenic animals in *Panagrellus redivivus*, by following the same protocols as used in *C. elegans*. Nonetheless, molecular and genetic techniques for non-*C. elegans* nematodes are generally being developed by the research community, such as in the Diplogasteridae species *P. pacificus* (Srinivasan, Sinz et al. 2002; Srinivasan, Sinz et al. 2003; Kenning, Kipping et al. 2004), and will likely become increasingly useful in the future.

Overall, this study demonstrated the potential utility of comparative studies in nematode behaviors. Comparative studies in nematodes have already been successfully used for investigating the evolutionary mechanisms of animal development (a few examples include Sommer 1997; Felix, De Ley et al. 2000; Dichtel, Louvet-Vallee et al. 2001).

Similarly, I believe that *C. elegans* and related nematodes can be of value for studying how behaviors evolve.

APPENDIX A

Videos of pharyngeal behaviors in free-living soil nematodes

***Caenorhabditis elegans* N2 (Rhabditidae family)**

Rhabditidae_N2_63x.mpg

Rhabditidae_N2_100x.mpg

***Mesorhabditis longespiculosa* DF5017 (Rhabditidae family)**

Rhabditidae_DF5017_63x.mpg

Rhabditidae_DF5017_100x.mpg

***Pristionchus pacificus* PS312 (Diplogasteridae family)**

Diplogasteridae_PS312_63x.mpg

Diplogasteridae_PS312_100x.mpg

***Cephalobus sp.* DWF1301 (Cephalobidae family)**

Cephalobidae_DWF1301_63x.mpg

Cephalobidae_DWF1301_100x.mpg

***Panagrolaimus sp.* PS1159 (Panagrolaimidae family)**

Panagrolaimidae_PS1159_63x.mpg

Panagrolaimidae_PS1159_100x.mpg

***Teratocephalus lirellus* PDL0011 (outgroup species)**

Teratocephalus_PDL0011_63x.mpg

All videos accessible at:

http://eatworms.swmed.edu/~alan

APPENDIX B

Videos of peristalsis defects in *ceh-28(tm1258)* mutants

Hour 0 of development (normal peristalsis)

tm1258_0hr.mpg

Hour 5 of development (slightly defective peristalsis)

tm1258_5hr.mpg

Hour 10 of development (defective peristalsis)

tm1258_10hr.mpg

Hour 15 of development (very defective peristalsis)

tm1258_15hr.mpg

Hour 20 of development (very defective peristalsis)

tm1258_20hr.mpg

All videos accessible at:

http://eatworms.swmed.edu/~alan

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VITAE

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