THE NEUROMOLECULAR MECHANISMS THAT COORDINATE FOOD AVAILABILITY WITH *C. elegans* MALE SEXUAL BEHAVIOR

A Dissertation

by

TODD R. GRUNINGER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2008

Major Subject: Genetics

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ABSTRACT

The Neuromolecular Mechanisms That Coordinate Food Availability with *C. elegans* Male Sexual Behavior. (August 2008)

Todd R. Gruninger, B.S., Texas A&M University

Chair of Advisory Committee: Dr. L. Rene Garcia

Organisms must coordinate behavioral and physiological responses to changing environmental conditions. In the nematode *C. elegans*, the presence or absence of food in the environment affects many metabolic and behavioral responses, including fat homeostasis, lifespan, and male mating. Specifically, male mating behavior normally occurs when a well-nourished male encounters a hermaphrodite, and is repressed if the male is under-nourished. To understand how environmental changes influence the drive to carry out specific behavioral tasks, I used *C. elegans* male mating as a model. Previously, mutants were isolated that display male mating behavior at inappropriate times, i.e. in the absence of mating cues. Loss of function mutations in the ERG K^+ channel, UNC-103, results in spontaneous seizures of the male sex muscles. Interestingly, I found that food deprivation can suppress *unc-103(lf)*-induced seizures, suggesting that pathways activated under this environmental condition can suppress the excitability of the mating circuit.

Using molecular, genetic, and behavioral assays, I identified sensory and molecular mechanisms that reduce sex-muscle excitability under food-deprived

conditions. I found that mutations that affect the muscular feeding organ, the pharynx, phenocopy the effects of food deprivation, and reduce sex-muscle excitability. I demonstrated that mutations in the pharyngeal muscle protein, tropomyosin, cause the pharyngeal neurosecretory motor neurons (NSMs) to increase pharyngeal excitability and reduce sex-muscle excitability. Additionally, I found that olfactory neurons (AWCs) with sensory cilia exposed to the environment are up-regulated in the absence of food stimuli, and also send inhibitory signals to the sex muscles. To determine how chemosensory and pharyngeal neurons in the head can signal to the genitalia, I hypothesized that one mechanism could be via secretion of metabolic hormones. To test this, I examined loss-of-function mutations in the insulin-like receptor, DAF-2, which is known to regulate many behavioral and physiological responses to food. I demonstrated that DAF-2 activity in the sex muscles is required for food-deprivation suppression of *unc-103(0)*-induced seizures. I then identified components of a novel-insulin-like/DAF-2 signaling pathway that reduces excitability. Specifically, I propose that ligand binding to DAF-2 activates PLC-γ and leads to increased cystolic Ca^{2+}. This Ca^{2+} influx activates CaMKII, which can phosphorylate/activate EAG-like K^{+} channels, thereby reducing cell excitability.

ACKNOWLEDGEMENTS

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I am also very thankful for the love and support of my parents and family during my work. I am especially grateful to my wife, Wendi, who has supported me through the highs and lows of my research career. She was always willing to listen, and understanding of the long days and the extra time necessary to complete my research.

NOMENCLATURE

ERG	*ether-a-go-go* related gene
EAG	*ether a-go-go* gene
CaMKII	calmodulin-dependent kinase II
ACh	acetylcholine
ARE	arecoline
LEV	levamisole
UNC-103	homolog of ERG K^+ channel
LEV-11	homolog of tropomyosin
DAF-2	homolog of insulin/IGF receptor
PLC-3	homolog of phospholipase C-γ
UNC-43	homolog of CaMKII
EGL-2	homolog of EAG K^+ channel

TABLE OF CONTENTS

CHAPTER Page

CHAPTER Page

Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

Coordination of behavioral and physiological responses to food availability

Both vertebrates and invertebrates encounter environments where food availability fluctuates and must endure periods of food deprivation. As a result, animals have evolved physiological responses to ensure survival until a food source is located. Responses to food deprivation include both behavioral and metabolic changes, such as promoting food-foraging behavior, mobilizing fat stores, and increasing life-span. In addition to activating life-prolonging responses, organisms must also repress sensory-motor circuits regulating other behaviors, such as reproduction. A better understanding of these adaptive responses will therefore not only elucidate how external cues influence behavioral outputs, but could also lead to improved treatment of abnormal metabolic and motor output.

During my graduate studies, I have been interested in understanding how environment influences behavioral output. Specifically, my goal was to obtain a better understanding of the neurobiological principles that explain why different environmental stimuli elicit specific behavioral responses. As a first step to addressing this broad question, I focused on a specific behavioral response to environmental change that I

This dissertation follows the style and format of PLoS Genetics.

hypothesized is present in most sexually reproducing organisms; the effect of food satiation on sexual libido. Although it is common to hear advertisements about foods such as oysters and chocolate that can supposedly enhance sex drive (i.e. aphrodisiacs), not much is known on how changes in diet can affect sexual and other motivational states in humans and lower animals. However, there has been significant research investigating the mechanisms that regulate other responses to food deprivation, specifically those that address a specific tissue need such as feeding and metabolic responses.

An organism's experience of food consists of multiple sensory cues, including odor, texture, and temperature. As a result, in addition to glucose levels, feeding and metabolic responses are also dependent on the sensory perception of food. For example, studies in humans have shown that sensory cues from food have significant effects on satiety and food intake, independent of ingestion [1]. These effects include sensory responses from chewing food which can enhance intestinal and gastric responses, as well as olfactory- and visual-mediated satiety [1-5]. Interestingly, two physiological responses to food studied in *Drosophila* and *C. elegans*, longevity and metabolism, are also regulated by olfactory and chemosensory neurons [6-9]. Taken together, these observations suggest that sensory perception of food in the environment, and not just intake of nutrients, can directly regulate physiology. Therefore, the effects of food deprivation on sensory-motor circuits controlling sexual behavior may involve a complex response to both internal metabolic and external environmental changes sensed by the nervous system.

In mammalian model systems, such as rodents, food restriction not only results in changes in metabolism leading to increased lifespan and stress resistance, but can also mediate changes in excitable cells, such as reducing the occurrence of epileptic seizures [10-18]. Epilepsy has been reported to affect over 50 million people worldwide [19], and much research has focused on pharmacological treatment of the main symptom of the disease, seizures. Over 80 years ago, the ketogenic diet, which is supposed to mimic the biochemical changes induced by fasting, was formulated as a treatment for patients with intractable seizures [20]. Although not much is known about how these diets improve control of seizures, recent work in rodents suggest that caloric restriction appears to decrease the probability of neurotransmitter release in presynaptic neurons of the dentate gyrus network [21]. These results support the idea that food restriction can directly modulate the excitability of neuronal circuits in vertebrates; however, the exact signaling mechanisms leading to this response still remain elusive.

Neuroscience studies in vertebrates rely heavily on electrophysiological, pharmacological, and Ca^{2+} imaging techniques. Although forward genetic approaches have recently been applied to vertebrate models [22,23], the sheer number of neurons involved, and long generation times make complete analysis of the molecules regulating behavioral circuits difficult. In contrast, the ease of genetic manipulation and unbiased forward genetic approaches in invertebrate systems such as *Drosophila* and *C. elegans* have identified many molecular components that govern sensory-motor responses in higher organisms [24-26]. Furthermore, the simpler nervous systems of invertebrate organisms are more amenable to circuit analyses at the technical and conceptual levels.

As a result, studies in invertebrate models can identify important signaling molecules more readily and can be used to direct further study in higher model systems. For example, studies in *C. elegans* and *Drosophila* have linked insulin-like signaling to food-deprivation responses such as increased life span and stress-resistance [27-29]. This has led to reverse genetic studies in vertebrate model systems, which demonstrated that insulin and insulin-like growth factor I (IGF-I) also regulate these responses in vertebrates [30,31].

Insulin's most widely studied role is its regulation of the body's ability to handle food by internalizing glucose. However, the finding that insulin can cross the blood-brain barrier and that insulin receptors are expressed throughout the vertebrate nervous system have led to the investigation of insulin's role in modulating behavior [32-35]. In *Drosophila*, insulin-like signaling has been demonstrated to regulate food preference and food foraging behaviors [36,37]. Similarly, in *C. elegans*, insulin-like signaling appears to regulate behavioral responses to food, including thermotaxis to temperatures previously associated with food [38] and associating salt with food-deprived conditions [39]. These studies suggest that insulin-mediated signaling pathways can regulate excitable circuits controlling behavior, and may coordinate metabolic and behavioral responses to food deprivation.

For this dissertation, I use *C. elegans* male mating behavior as a model to dissect how environment influences behavioral tasks not associated with fulfilling a particular tissue need. Specifically, I dissect the effects of food deprivation on male mating behavior and on spontaneous sex-muscle seizures caused by mutations in the ERG-like

K^+ channel, UNC-103. I provide evidence that food deprivation suppresses male sexual behavior and spontaneous *unc-103(lf)*-seizures via insulin-like signals generated from the pharyngeal feeding organ and chemosensory neurons. Additionally, I describe a novel insulin-like signaling pathway existing in muscles that can suppress cell excitability.

C. elegans as a model organism to study sensory-motor responses

The free-living soil nematode *Caenorhabditis elegans* was first introduced as a model organism by Sydney Brenner in 1960 due to its many genetic advantages, including short life cycle, large brood size, and hermaphroditic means of reproduction [40]. There are two sexes in *C. elegans*, hermaphrodite and male, with each having a transparent body length of approximately 1mm (hermaphrodites are slightly larger than males). Both sexes share most components of their nervous system, however there are some sex-specific differences. The hermaphrodite contains 302 neurons, in which 8 are sex-specific, while the male contains 387 neurons with 89 of them being male-specific [41,42]. The wiring of the hermaphrodite nervous system has been completed, and there has been significant progress on the male circuitry, mostly led by Scott Emmons, which is expected to be completed in the near future (www.wormatlas.org) [42]. Combined with the powerful genetic tools, the details of synaptic connectivity in individual circuits governing different motor outputs allow us the complete analysis of how an organism

can regulate behavioral responses, including both the neuronal and molecular details involved.

C. elegans male mating behavior

C. elegans male mating behavior initiates when sensilla in the male tail contact a hermaphrodite (Figure 1). The male then presses the ventral portion of his tail against the hermaphrodite's cuticle and begins scanning for her vulva. If the male reaches the end of the hermaphrodite without sensing the vulva, he performs a ventral turn and scans the other side. Once he locates the vulva, he stops and attempts to insert his copulatory spicules into his mate. Spicule insertion is achieved by rapid contraction of his sex muscles, which consist of two retractor muscles and two protractor muscles attached to each of his two spicules. Once he has breached the vulva, he maintains a tonic contraction of his sex muscles, which keeps his spicules fully inserted in the vulva and allows sperm to be transferred into his mate [41,43-46]. Since mating consists of multiple sub-steps regulated by different neuromuscular circuits, it is possible that food deprivation could act on multiple circuits to suppress the behavior. For this work, I focused on the spicule insertion step of male mating since the key tissues and molecules that regulate this output under normal conditions have previously been identified.

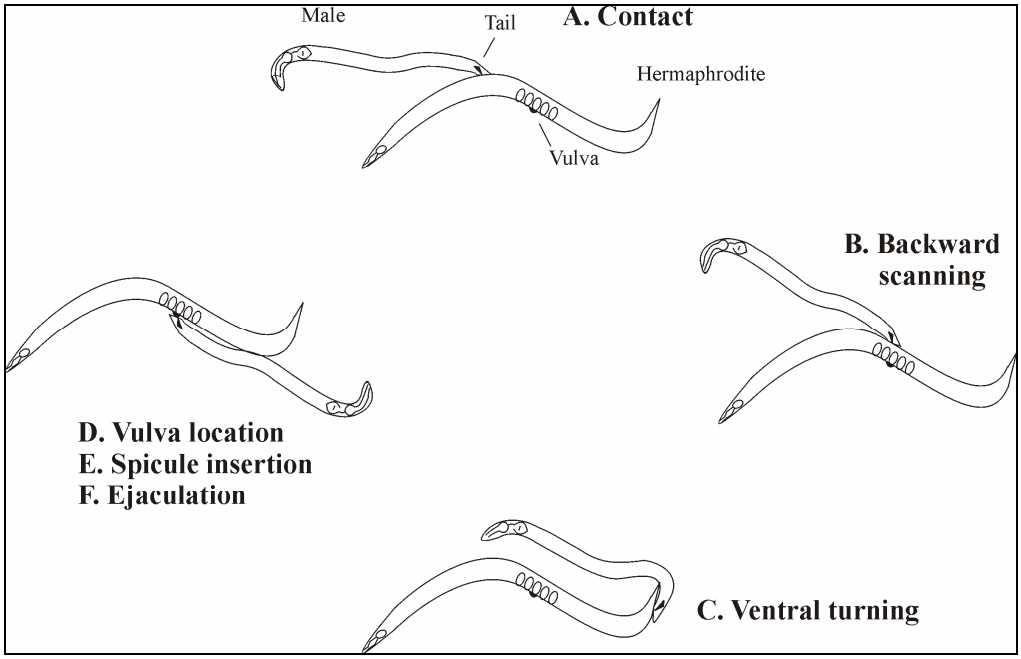

Figure 1. Steps in *C. elegans* male mating behavior.

Previous cell-ablation and genetic analyses on the male circuitry have revealed the basic neuronal circuit and signaling molecules used to regulate sex-muscle output (Figure 2A) [46]. Spicule insertion behavior initiates when the hook sensillum and postcloacal sensilla contact the hermaphrodite vulva. The neurons with sensory endings

in the hook (HOA, HOB) and post cloacal sensila (PCB, PCC) stimulate the protractor muscles to contract and relax at a frequency of 7-9Hz, by releasing acetylcholine (ACh). ACh from these neurons activates ACh receptors on the muscles, and causes influx of Ca^{2+} through the ryanodine receptor (RyR), UNC-68. RyRs mediated the release of calcium from the sarcoplasmic reticulum to induce muscle contraction. Once the tips of the spicules have partially penetrated the vulva lips, the SPC sensory/motor neuron also releases ACh. In contrast to the post cloacal and hook neurons, activation of ACh receptors by the SPC neuron leads to downstream activation of L-type voltage-gated Ca^{2+} channels (L-VGCC), and tonic contraction of the sex muscles via influx of extracellular calcium (Figure 2B). It is possible that the amount or duration of calcium signaling via L-VGCC causes the protractor muscles to fully contract and remain tonic [46]. The mechanisms used to suppress male mating during periods of food deprivation may ultimately act to repress these signaling pathways.

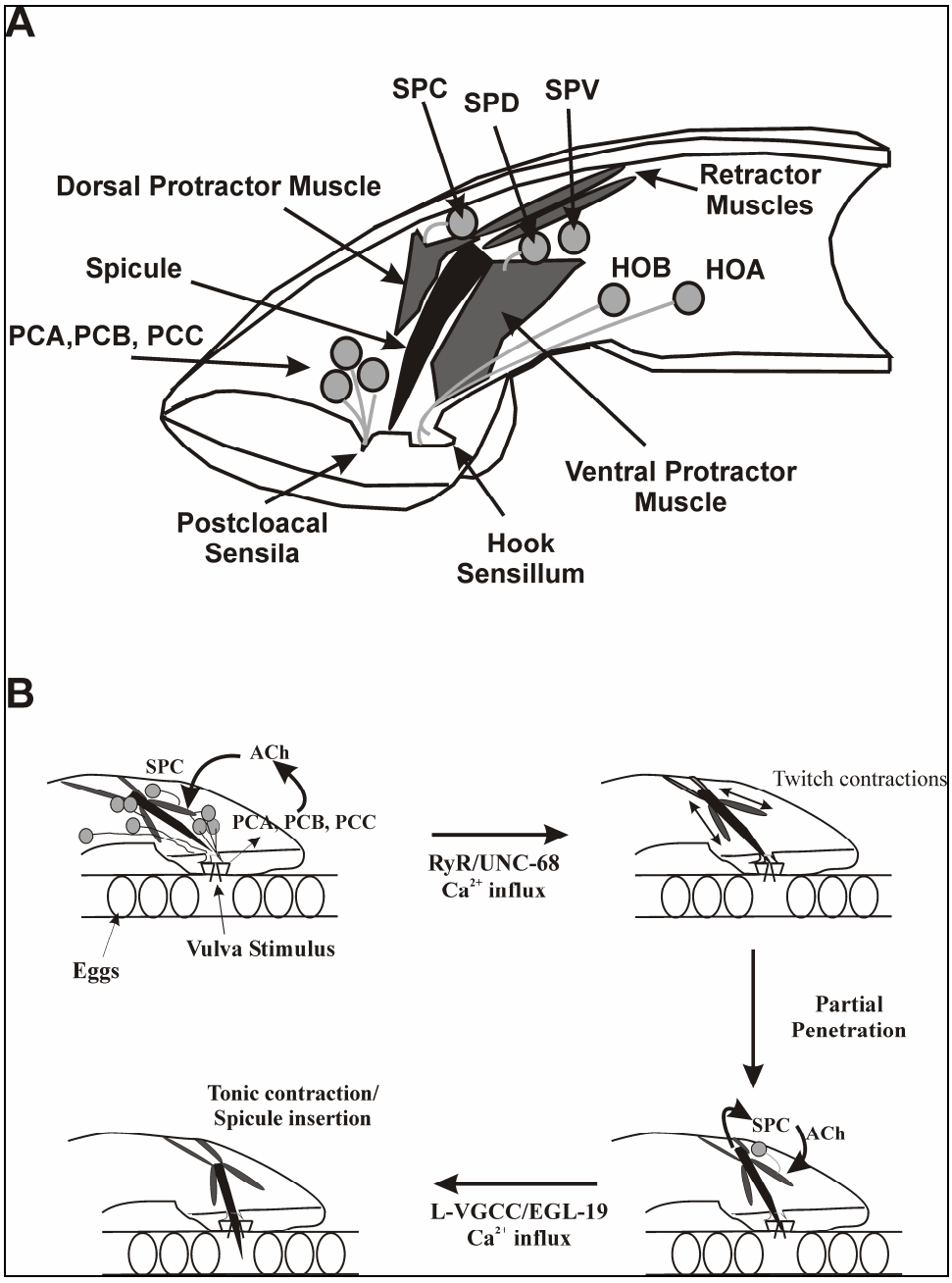

Figure 2. Anatomy and regulation of the male genitalia.
(A) The male muscles and sensory structures used during spicule insertion behavior.
(B) The current model for spicule insertion behavior. Vulva stimulation causes the PCB, and PCC neurons to secrete ACh at their synapses with the protractor muscles. ACh from these neurons binds to levamisole- and nicotinic-sensitive ACh receptors and results in activation of RyR/UNC-68 Ca^{2+} channels, causing rapid twitch contractions. Once the spicules have partially penetrated the vulva, the SPC neuron secretes ACh at its synapse with the protractor muscles. ACh from SPC binds arecoline- and levamisole-sensitive ACh receptors, which leads to activation of VGCC/EGL-19 Ca^{2+} channels and tonic contractions of the muscles.

ERG K^+ channels regulate sex-muscle excitability

The execution of sensory-motor output is largely dependent on membrane polarization of excitable cells. As a result, defects in membrane channels can lead to irregular motor output and a variety of medical conditions. One member of the ether-a-go-go (EAG) class of voltage-gated K^+ channels, encoded by the human *ether-a-go-go related gene* (*hERG*), has been shown to be vital in regulating cardiac muscle contractions by repolarizing the cardiac action potential [47-49]. In contrast to other voltage-gated K+ channels, ERG channels display slow activation and rapid inactivation kinetics, ensuring that the channel passes little current during membrane depolarization. However, during a subsequent repolarization, the channel rapidly recovers from inactivation and inhibits subsequent depolarizations, protecting against cardiac arrhythmias caused by spontaneous firing [50,51]. Due to the significance of this protein, there is ongoing research into the channel's kinetics and pharmaceutical agents are routinely pre-screened for hERG channel blockage [52-55].

Previous work in *C. elegans* has demonstrated that UNC-103, the ortholog of the human ERG voltage-gated K^+ channel (HERG), is required to suppress sex-muscle contractions in the absence of mating cues [56]. Similar to blockage of hERG, loss-of-function alleles of *unc-103* cause males to suffer spontaneous seizures of their sex muscles, and display spicule protraction in the absence of mating cues. The *n1213* allele of *unc-103* (*unc-103(0)*), causes 30-40% of males to display precocious spicule protraction, while the *sy557* dominant-negative allele of *unc-103* (*unc-103(dn)*), results

in 60-80% of males displaying the defect. The higher penetrance of the dominant-negative allele suggests that it may be interfering with other channels that can counteract increases in cell excitability [56]. It is likely that *unc-103(lf)* alleles cause spontaneous protraction by increasing the excitability of the sex muscles. If UNC-103 suppresses spontaneous sex-muscle contractions by acting in the sex muscles to reduce excitability, the *unc-103(lf)* phenotype could be used as a proxy to study how perturbations affect sex-muscle excitability. A cartoon model for how a voltage-gated K^+ channel could regulate sex-muscle excitability is given in Figure 3. Normally, when the muscle is relaxed, the cell membrane is polarized, consisting of more negatively charged ions on the intracellular side than the extracellular side. Upon stimulation by a neuron, a neurotransmitter such as ACh can bind a ligand-gated channel, causing it to open and allow positive ions to flow into the cell. This voltage-change/membrane depolarization can be sensed by voltage-gated calcium channels, causing them to open and allow calcium influx. Calcium can then activate myosin-actin cross bridge cycling, and induce muscle contraction. The role of voltage-gated K^+ channels such as UNC-103/ERG, is to negatively regulate membrane depolarizations by opening and allowing positively charged K^+ ions to flow out of the cell. This then repolarizes the membrane and results in closure of the voltage-gated Ca^{2+} channels, and with no more Ca^{2+} influx to activate the muscle contractile proteins, the muscle is relaxed.

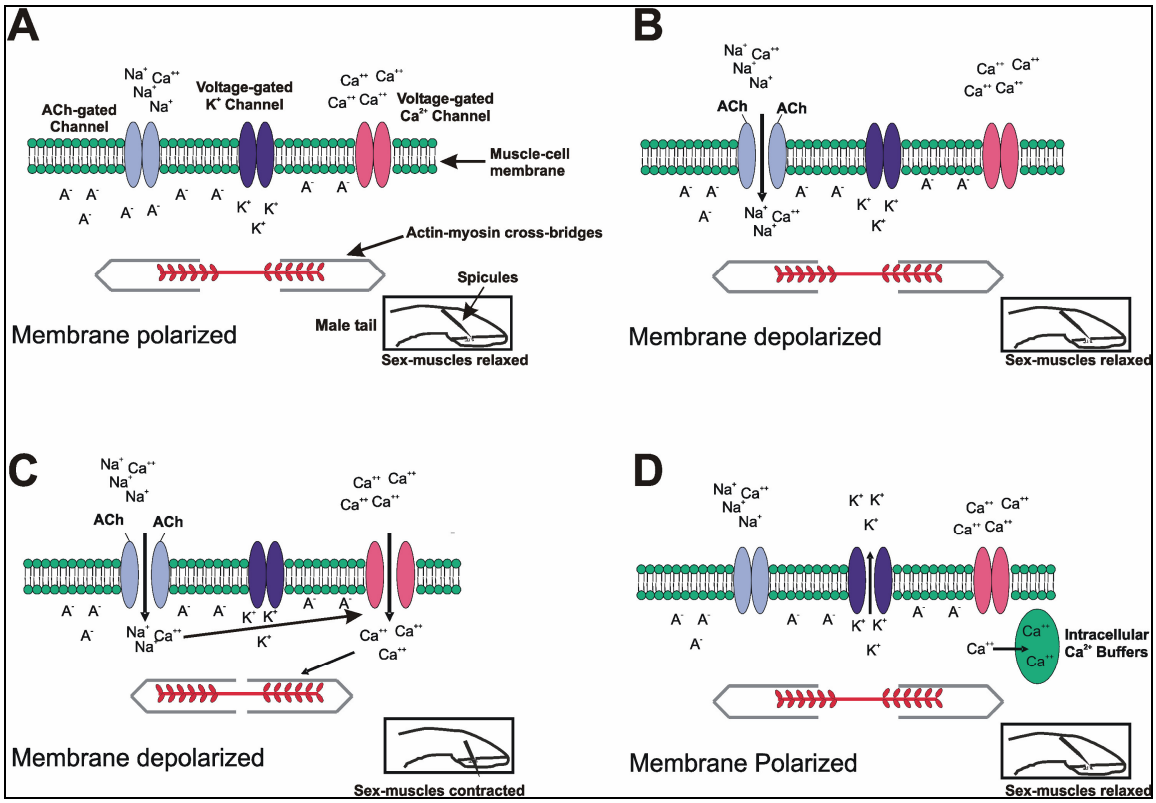

Figure 3. Cartoon model of how ERG-like K^+ channels/UNC-103 regulate muscle excitability.
(A) Before stimulation the membrane is polarized.
(B) ACh-activated channels open in response to ACh binding and allow the influx of positive Ca^{2+} and Na^+ ions.
(C) The resulting voltage change activates voltage-gated Ca^{2+} channels and allows more Ca^{2+} influx which can bind troponin/tropomyosin, and initiate muscle contraction.
(D) Voltage-gated K^+ channels negatively regulate this by allowing K^+ efflux and repolarizing the membrane. The lower right-hand panel depicts what happens to the male's copulatory spicules when the sex muscles are contracted or relaxed.

Dissertation objectives

The objective of this dissertation is to identify novel signaling mechanisms used to coordinate behavior with changing environmental conditions, using *C. elegans* male sexual behavior as a model.

In Chapter II, I give the detailed materials and protocols used for the experiments performed in Chapter III-V. In Chapter III, I describe and quantify the suppressing effects that food deprivation has on male mating behavior. I also demonstrate that food deprivation can suppress mutant-induced sex-muscle seizures caused by loss of ERG-like K^+ channel function. In Chapter IV, I provide evidence that food-deprivation suppression of mutant-induced seizures can occur through two mechanisms, via signals from the neuromuscular feeding organ, the pharynx, and via chemosensory neurons with cilia exposed to the environment. In Chapter V, I identify an insulin-like signaling pathway as a possible mechanism used by pharyngeal and chemosensory neurons in the head to influence genital circuits in the tail. Specifically, I find that insulin-like receptor signaling in the sex muscles is required for food-deprivation suppression of seizures, and this signaling does not involve the canonical FOXO/DAF-16 transcription factor. Instead, I describe a novel pathway that includes PLC-γ, CaMKII, and EAG-like K^+ channels.

CHAPTER II

EXPERIMENTAL PROCEDURES

Strains

All strains contain *him-5(e1490)* (LGV) [57] and were maintained according to [40]. The following strains were used in this study: LGII: The *plc-3(sy698)* strain used in this study is PS5109 described previously in [58] and graciously provided by Cheryl Van Buskirk; LGI: *lev-11(x12)* [59], *lev-11(rg1)* [60]; LGIII: *daf-2(e1368)* [61], *daf-2(m41)* [62], *unc-103(n1213)* [63], *unc-103(sy557)* [56], *unc-64(e246)* [40], and *pha-1(e2123)* [64]; LG IV: *egl-19(n2386gf)* [65], *unc-43(sy574)* [56,66], *osm-9(ky10)* [67]; LGV: *egl-2(n693)* [68]; LGX: *osm-5(p813)* [69].

Assay for protaction constitutive males

Approximately 20-30 L4 males were separated and allowed to develop on one of three types of plates: NGM plates seeded with OP50, NGM plates without OP50, or aztreonam-LB/NGM plates seeded with pre-azetreonam-treated OP50 and scored 15-20 hours later for spontaneous spicule protraction. The protocol for aztreonam-NGM plates and aztreonam treatment is given below. Males that crawled up on the side of the plate and dried up were not scored. To minimize suicidal males, we used an 8M glycerol ring

around the edge of plates for food-deprivation assays. The glycerol ring acted as a repellent to keep males off the sides of the plates; the 8-M glycerol ring induced no other significant changes in behavior (data not shown). To measure the effects of food deprivation, we separated late-L4 males to a clean NGM plate with no bacteria and allowed them to crawl away from any bacteria transferred. Males were then picked up by mouth pipette washed with M9 buffer, and transferred either to a microfuge tube containing 20µl S-medium with or without *E. coli* OP50 for liquid-starvation assays, or to food-deprivation assay plates. For *daf-2(e1368)* lines, males were raised at 20ºC until L2-L3 stage and then kept at 25ºC for food-deprivation assays, unless otherwise noted. For *daf-2(m41)* lines, males were raised at 15ºC until L2-L3 stage and then raised and food-deprived at 25ºC. For all assays, males were picked from non-crowded and non-contaminated plates.

Mating observations

We observed mating with a Stemi SV11 dissecting microscope (Zeiss, Thornwood, NY). We placed 10 *unc-64(e246)* hermaphrodites and one male on a 10mm plate seeded with 5mm OP50 lawn. To assay each male's drive to mate, we recorded the number of hermaphrodites a male encountered before he initiated mating behavior. Males who required three or more encounters were considered defective. A male was considered defective in turning if he failed to complete a successful turn within three attempts of reaching the end of the hermaphrodite's cuticle. A male was considered

vulva location defective if he required three or more contacts with a hermaphrodite's vulva before attempting to insert his spicules.

For mating efficiency assays, we separated L4 males from hermaphodites and placed them on one of the three different feeding conditions, similar to the above assay. The next day, we then placed one male with one *pha-1(e2123)* hermaphrodite on 3.5cm NGM plate that was seeded with a .5cm diameter lawn of log-phase *E. coli* OP50 15hrs prior to the assay. *pha-1(e2123)* is a temperature-sensitive mutation that allows growth at 15ºC, but is lethal at 25°C. Males were allowed to mate for 2hrs and then removed from the plate. Plates were then incubated at 25°C for 48hrs and then scored for the presence or absence of *pha-1(+)* growing F1 progeny.

Aztreonam-treated OP50 (inedible food)

OP50 cultures were grown to log growth in LB at 37°C with rigorous shaking. Once the cells reached log growth, aztreonam (Sigma) was added to a final concentration of 10µg/ml, and the cells were grown an additional 3hrs with minimal shaking to minimize damage to the bacterial filaments. Cells were then spotted on LB plates containing NGM ingredients and 10µg/mL aztreonam. Azteonam-treated bacteria and plates were used fresh (the same day), as plates older than 2 days had normal length bacteria, likely due to the drug being exhausted.

Assay for the effects of AWC odorants on spicule protraction

To phenocopy the effect of the AWC ablation and *Podr-3:unc-103(gf)* experiments, we used odorants previously shown to reduce AWC activity. We used Sephadex G-50 beads (20-80µm) (Sigma, www.sigmaaldrich.com) and acetone as a vehicle for the worms to chemotax to and contact. Specifically, we used a sterile M9 solution containing 10-3 butanone, 10-4 isoamyl alcohol, 10-7 2,3-pentanedione, 10-4 benzaldehyde, and 30mg/ml Sephadex beads. 20µl of this solution was spotted on a sterile NGM plate along with 1µl of .4M sodium acetate. As a control, we spotted 20µl of a M9 solution containing only 30mg/ml Sephadex beads along with 1µl .4M sodium acetate on a sterile NGM plate. The sodium acetate ensured that the worms remained near the soaked beads, and showed no effects on spicule protraction mutants when used in isolation.

Pharmacology

We dissolved levamisole (purchased from ICN Biomedicals Inc, Aurora, Ohio) in distilled water to make a 100mM stock solution. The stock was then serially diluted in water as needed. 1ml of the drug was added to a three-well, Pyrex titer dish, and 10 <24 hr virgin adult males were placed in the drug. We observed the males for 2 minutes with a Wild M5A microscope. The males were considered resistant to the drug if they were not paralyzed after 2 minutes. To measure the sensitivity of the spicule muscles to

levamisole, five virgin adult males were added to the drug and observed for 5 minutes. We scored the males as sensitive to the drug if they kept their spicules protracted for at least 10 seconds. For effects of food-deprivation on arecoline sensitivity, L4 males were separated from hermaphrodites and placed on either NGM plates seeded with OP50 or NGM plates with no OP50 for 15-20hrs, and then scored for drug sensitivity. Arecoline was obtained from Indofine Chemical Company (www.indofinechemical.com). Curve fits, EC90 and EC50 concentrations were generated using GraphPad Prism 4 software (GraphPad Software, Inc.).

RNA-mediated interference (RNAi)

To generate dsRNA for the TnT genes, we PCR-amplified individual exons of *tnt-1*, *tnt-2*, *tnt-3*, and *tnt-4* from N2 genomic DNA. We then flanked each exon with T7 promoter sequences using T7 primers. Primer sequences are listed in the appendix. PCR products were then used for *in vitro* transcription with a T7-MEGAshortscript™ kit from Ambion. Recovery of purified dsRNA was obtained following the protocol supplied with the kit. The final RNA concentration for each reaction was ~ 5µg/µl. To generate double-stranded RNA (dsRNA) for PLC-2, we PCR amplified a region of exon 9 using the primers FT7plc2 and RT7plc2 flanked by T7 promoter sequences. For PLC-3, we amplified exon 5 using the primers FT7plc3 and RT7plc3 flanked by T7 promoter sequences. The resulting PCR products were then used as a template for *in vitro*

transcription with a T7-MEGAshortscript kit from Ambion (Austin, TX). The final RNA concentration of each reaction was ~2µg/µl.

To perform RNAi on the TnT genes, we placed L4 stage males in microfuge tubes containing a solution of 5µl of dsRNA, 13µl S-medium, and 2µl concentrated OP50 as the food source. Control worms were placed in a solution containing only S-medium and OP50. After 24hrs, we removed the adult males to a fresh NGM plate and immediately scored them for the Prc phenotype. To perform RNAi on the PLC genes, we placed L4 males in microfuge tubes containing 5µl of dsRNA, and 15µl of S-Medium. For controls with food, 2µl of concentrated OP50 was added as a food source along with 5µl dsRNA and 13µl S-medium. After 15hrs, males were removed from the microfuge tubes and scored for spontaneous protraction of their spicules.

Plasmids

All primers and sequences are listed in the Appendix (Tables A-1 and A-2). pDG9 contains GFP expressed from sequences 3.5kb upstream of the first *lev-11* exon. We PCR-amplified these *lev-11* sequences and then blunt end-ligated this fragment into the *Sma*I site of pPD95.77 (plasmid courtesy A. Fire, Stanford University School of Medicine). pTG13 contains CFP expressed from *lev-11* sequences including 1.3kb upstream of exon 3b to exon 6. We PCR amplified these sequences using primers flanked with attB sites and recombined the product into pDONR221, a donor vector from Invitrogen, to produce pTG12. pTG13 was then generated by recombining pTG12

into pGW77C. pGW77C is a plasmid derived from pSX77CFP (plasmid courtesy of Nadeem Moghal, Huntsman Cancer Institute University of Utah) containing the Gateway cloning cassette. pDG9 was injected into the germline of *him-5* hermaphrodites at a concentration of 5ng/µl and pTG13 was injected at 2 ng/µl [70]. Three lines from each injection were analyzed for *lev-11* expression. To generate pLR10, we PCR-amplified an 8.7kb region between the first *unc-103* exon and the preceding gene C30D11.2. We then blunt end-ligated this PCR fragment into the *Sma*I site of the plasmid pSX77YFP, a vector derived from pPD95.77 that contains YFP in place of GFP (plasmid courtesy of Nadeem Moghal, Huntsman Cancer Institute University of Utah). We injected a mixture containing pLR10 (10ng/µl), pBX1 (100ng/µl), and pUC18 (90ng/µl) into the germline of *pha-1*; *him-5* hermaphrodites [70,71]. Three transmitting lines were then analyzed for *unc-103* expression. Cells were identified using drawings found in Sulston et al. 1980 and White et al. 1986. To observe *unc-103* sex muscle expression, we first PCR-amplified ~ 16kb of DNA containing the 8.7kb region upstream of the first *unc-103* exon and all coding sequences excluding the stop codon. We cut the ends of this PCR fragment with *Nhe*I and ligated it to *Xba*1-digested pSX77YFP, so that the *unc-103* coding sequence was in frame with YFP. We found this construct to be unstable in *E. coli*; therefore, we injected the ligation mixture directly into *C. elegans*. To generate transgenic worms, we co-injected the 16kb *unc-103- Xba*1-digested pSX77YFP mixture (200ng/µl) with pBX1 (100ng/µl) into *unc-103(n1213) pha-1*; *him-5* hermaphrodites. We obtained seven transmitting lines. All lines were fluorescent, suppressed for the Prc phenotype, lethargic, and had reduced egg-laying

behavior, suggesting that the transgene was overexpressed. We analyzed worms carrying the transgene array *rgEx20*. When the ligation mixture was injected at 100ng/µl, the synthetic phenotypes were reduced, but the fluorescence was also too dim to identify cells.

To generate pTG7, we PCR-amplified a 6.2kb fragment of DNA that covers 3kb upstream of the start codon and exons 1 through 5 of *tnt-4*. pPD95.79 (plasmid courtesy A. Fire, Stanford University School of Medicine) was cut with *Apa*I, *Fsp*I, and *Hind*III, and the 2000bp fragment was retained. pBR322 was cut with *Ava*1 and *Hind*III; the 2953bp fragment was retained and blunt end-ligated to the pPD95.79 fragment above to make pTG5. We then blunt end-ligated the *tnt-4* PCR fragment into the *Sma*I site of pTG5 to make the plasmid pTG7. GFP is fused in frame to the last exon of the smaller of the two reported *tnt-4* isoforms (www.wormbase.org). pTG7 (20ng/µl), pBX1 (100ng/µl), and pUC18 (80ng/µl) were injected into the germline of *pha-1; him-5* hermaphodites [70,71]. We analyzed 20 mid-L4 and 20 adult males from two transgenic lines for *tnt-4* expression.

To generate pTG11, we amplified sequences 1kb upstream of the *tph-1* start codon [72]. This sequence was re-amplified using the same primers flanked with attB sites. This was then recombined into pDONR221 to generate pTG9. pTG9 was then subsequently recombined into pGW77C to make pTG11. pTG11 (50ng/µl), pBX1 (100ng/µl), and pDONR221(Invitrogen) (50ng/µl) were injected into the germline of *pha-1; him-5* hermaphrodites.

To place promoters upstream of G-CaMP, the MCS, synthetic intron and *unc-54* 3' UTR was cut out of pPD49.26 (A. Fire) using ApaI (blunted with T4 DNA polymerase) and HindIII. This fragment was then cloned into the AvaI (blunted) and HindIII sites of pBR322 to make pTG24. A region containing G-CaMP was cut out of the vector pN1-G-CaMP1.3 [73] using NotI (blunted) and BglII, and cloned into the SmaI-BamHI sites of pTG24 to make pTG29. pBX1 was cut with XhoI (blunted) and XbaI, and the 7.3kb fragment that contains the *pha-1* promoter and gene was cloned into the EcoRI (blunted) and XbaI sites of pTG29 to generate pLR133. The Gateway Cassette C.1 (Invitrogen) was then blunt-end cloned into the XbaI site of pLR133 to generate pLR134. This allows various promoters to be recombined upstream of G-CaMP. To drive expression of G-CaMP in the sex muscles, the plasmid pLR21[74], which contains the *unc-1031E* promoter, was recombined with pLR134 using LR clonase (Invitrogen) to generate pLR136. To drive G-CaMP in *odr-3* expressing neurons, pTG70 (described below) was recombined with pLR134 LR clonase to generate pTG81.

Plasmids containing *unc-103* genomic DNA were created as previously described [74]. pLR62 contains *unc-103* genomic DNA and was used as template to generate the A331T mutation that exists in *unc-103(e1597gf)* using single-site mutagenesis. The addition of the *unc-103(gf)* mutation to pLR62 generated pLR73. To generate chemosensory promoters driving YFP and *unc-103(gf)*, we amplified promoter sequences flanked with attB sites and recombined each into the Gateway entry vector, pDG15 [74] using BP clonase (Invitrogen). pTG68 contains 3.8kb of *ocr-2* upstream

sequences, pTG70 contains 2.7kb of *odr-3* upstream sequences, pLR88 contains 1.2kb of *osm-12* upstream sequences, and pTG87 contains 1.9kb of *tax-2* upstream sequences. These promoter-containing plasmids were then recombined using LR clonase into pGW322YFP or pLR73, which contain attR recombination sites in front of YFP and *unc-103(gf)*, respectively. pTG81 contains *pha-1(+)* from pBX1 and the *odr-3* promoter driving G-CaMP. pTG71, pTG73, and pTG82 contain the *ocr-2*, *odr-3*, and *osm-12* promoters driving *unc-103(gf)*. pTG76, pTG78, and pLR89 contain YFP expressed from the *odr-3*, *ocr-2*, and *osm-12* promoters, respectively.

To generate *daf-2(+)* rescue constructs, the *daf-2* cDNA was amplified from a cDNA library using primers flanked with attB sites (Invitrogen). A 1.1kb region from pDONR221 containing an *att*P1 site, *ccdB* gene, chloramphenicol resistance gene, and *att*P2 site (Invitrogen) was PCR amplified (primers ATTP1 and ATTP2) and cloned into the SmaI site of pTG24 to create pTG38. The *daf-2* cDNA was then recombined into pTG38 to make pTG39. The *daf-2* cDNA was sequenced in pTG39 and 4 mutations were fixed using site-directed mutagenesis, to make pTG53. To place promoters in front of *daf-2*, the 1.1kb region of pDONR221 was cloned into the XbaI site of pTG53 to generate pTG54. Promoter regions for *unc-103E*, *aex-3*, *gtl-1*, *lev-11*, *tnt-4*, and *unc-103F* were amplified using the primers listed in Supplemental Table 1 and recombined into pTG54 using BP clonase to generate pTG57, pTG58, pTG59, pTG60, pTG61, pTG65, respectively. pBL71 contains *Punc-103E:unc-43(+)*, and its construction is described in [66].

To construct plasmids containing sequences upstream of *egl-2,* we PCR-amplified from genomic DNA, an 8.5 kb fragment using the primers ATTB1UPSEGL2 and ATTB2EGL2DWN. To generate the plasmid pLR11, we recombined this fragment into the gateway entry vector pDONR221 (Invitrogen) using BP clonase. To drive YFP expression from *egl-2* upstream sequences, we recombined the *egl-2* promoter region from pLR11 into the YFP-containing Gateway destination vector pGW322YFP [74] using LR clonase; this generated the plasmid pLR15.

Plasmids containing *egl-2* cDNA were constructed as follows. A *Hind*III to *Apa*I fragment from the plasmid pPD49.26, containing the multi-cloning site to the *unc-54* 3' UTR, was cloned into the *Hind*III-*Ava*I sites of pBR322 to generate pTG24 (plasmid pPD49.26 courtesy of A. Fire). The *egl-2* cDNA was PCR- amplified from a cDNA library using primers pstegl-2atg and kpnegl2tga. pTG24 was cut with *Kpn*I and partially digested with *Pst*I. The *egl-2* cDNA was digested with *Pst*I and *Kpn*I and cloned into the *Pst*I-*Kpn*I sites of pTG24 to generate pLR120. The *egl-2* cDNA in pLR120 was sequenced and compared to genomic *egl-2* DNA to confirm no changes to the amino acid sequence were induced by PCR. pLR120 was then cut with *Sph*I, and then blunted with T4 DNA polymerase. The Invitrogen Gateway reading frame cassette C.1 was then cloned into this site to generate pTG43. The *unc-103* promoter E and the *lev-11* promoter were introduced in front of the *egl-2*cDNA by recombining the plasmid pLR21 and pLR22 with pTG43 using LR clonase to generate pTG44 and pTG46, respectively.

To generate a PLC-3 promoter-YFP reporter, we amplified a region containing a 4.4kb upstream region of PLC-3 plus the first 12 codons with primers flanked with attb

sequences. This fragment was then recombined into pDG15 using BP clonase to generate pTG91. pTG91 was then recombined with pGW322YFP using LR clonase to generate pTG92.

To generate a GFP-tagged LGG-1, we first used the primers HIIIYFP and NRLGGYFP to amplify an 800bp fragment of pGW322YFP which contains YFP. We then used the primers NATTB2LGG and XBALGG to amplify a 2.3kb region of N2 DNA that includes the *lgg-1* gene and 3'UTR. We then used PCR sewing to combine the 2.3kb and 800bp regions using the HIIYFP and XBALGG primers. This fragment was then cut with XbaI and HindIII and cloned into the XbaI and HindIII sites of pTG24 to generate pLR139. We then blunt-end cloned the Gateway Cassette C.1 into the HindIII site of pLR139 to generate pLR141. This allows for various promoters to be recombined in front of LGG-1::YFP. pLR22 contains the *lev-11* promoter cloned into pDG15 [66,74]. pLR141 and pLR22 were then recombined using LR clonase to generate pTG90 (*Plev-11:LGG-1::YFP*).

Characterization of *unc-103* promoter and coding sequences

For characterization of the cDNA structure of *unc-103*, polyA mRNA was isolated from a population of mixed-staged *him-5(e1490)* worms [74]. cDNA from polyA mRNA was made using the Invitrogen 5'RACE System (Invitrogen, Carlsbad, CA) using the primer unc-103race2, which anneals to exon 4, and the 5' RACE primer supplied with the kit. Invitrogen Gateway attB sites were added to pooled 5'RACE

fragments via PCR using the primers unc-103race5 and unc-103race6. The resulting attB-linked 5'RACE fragments were then inserted into pDONR221 (Invitrogen) using BP clonase, and then transformed into *E. coli* DH5α. Individual clones were isolated and then sequenced. Primers used in this experiment are listed in the Appendix (Table A-2).

Transgenics

To rescue *lev-11(rg1)*, we generated tissue-specific rescue constructs. The *lev-11* gene has been reported to contain two groups of isoforms; those that express in the body-wall, anal, and sex muscle, and those that express in the pharyngeal muscle and intestine [75,76]. To omit the promoter and exons specific to the intestine and pharyngeal forms, we used 4 specific primers. Primer 1 and Primer 2 were used to amplify sequences containing 3.5kb upstream of the start codon down to exon 3a. Primer 2 also contains homology to the initial sequence of exon 4b. Primer 3 and Primer 4 were used to amplify sequences containing exon 4b down to 475bp of the 3' UTR. Primer 4 contains homology to the exon 3a specific sequences of Primer 2. The two PCR products were then annealed, extended, and subsequently amplified using primers 1 and 3. The body-wall, anal, and sex muscle specific construct (2ng/µl), pBX1 (100ng/µl), and the GFP marker plasmid pDG9 (5ng/µl) were injected into the germline of *lev-11(rg1); unc-103(sy557) pha-1; him-5* hermaphrodites. To rescue *lev-11(rg1)* in pharyngeal muscle and intestine, we PCR-amplified a 6kb DNA fragment from N2 DNA that contains 1.3kb of sequences upstream of *lev-11* exon 3b, exons 3b to 9c, and the 3'UTR [76]. We

injected the pharyngeal muscle and intestine specific construct at 200pg/µl with pBX1 (100ng/µl) and the *myo-2::*GFP plasmid, pPD118.33 (plasmid courtesy A. Fire, Stanford University School of Medicine). Injections that contained the pharyngeal/intestine construct at concentrations ≥200pg/µl resulted in larval arrest. We obtained seven lines for both sets of injections, *rgEx26* (body-wall/anal/sex muscle *lev-11(+)*) and *rgEx27* (pharyngeal/intestine *lev-11(+)*) were further analyzed.

To obtain *lev-11(rg1); unc-103(n1213); him-5* animals with *rgEx26* or *rgEx27*, we crossed *lev-11(rg1); unc-103(sy557) pha-1; him-5; rgEx26* and *lev-11(rg1); unc-103(sy557) pha-1; him-5; rgEx27* males into *pha-1; unc-103(e1597gf); him-5* hermaphrodites. F2 animals homozygous for *lev-11(rg1)* and *unc-103(e1597)* were kept, and hermaphrodites still expressing the GFP marker were then crossed with *lev-11(rg1); unc-103(n1213); him-5* males. We repulsed *unc-103(e1597gf)* to obtain *lev-11(rg1); unc-103(n1213); him5; rgEx26* and *lev-11(rg1); unc-103(sy557); him-5; rgEx27*; fluorescing transgenic males were then assayed for the Prc phenotype.

To obtain *unc-103* transgenic lines, DNA was co-injected with pBX1 (100ng/µl) into *pha-1(e2131) unc-103(n1213)* hermaphrodites. The pBX1 plasmid contains *pha-1(+)* and was used to select for transformants [77]. For *unc-103(gf)* and YFP constructs, all constructs were injected at 25ng/µl.

For the *daf-2(+)* rescue constructs, DNA was co-injected with a GFP-marker into *daf-2(e1368) unc-103(n1213)* hermaphrodites. The *daf-2* rescue constructs and GFP markers were combined with pUC18 to bring the final DNA concentration to 200ng/µl and the injection mixtures for each rescue were: pTG60 (50ng/µl) and pDG9 (5ng/µl);

pTG58 (50ng/µl) and pTG6 (20ng/µl); pTG57 (50ng/µl) and pDG9 (5ng/µl); pTG65 (50ng/µl) and pDG9 (5ng/µl); pTG59 (50ng/µl) and pTG6 (20ng/µl); pTG61 (50ng/µl) and pTG6 (20ng/µl). The GFP-markers were used to select for transgenic males and at least two independent lines were analyzed for each injection.

For rescue of *unc-43* mutants, pBL71, which contains *Punc-103E:unc-43(+)*, was injected at 10ng/µl along with 170ng/µl pUC18 and 20ng/µl of pBL66. pBL66 contains CFP expressed from the *gtl-1* promoter and was used to mark transgenic lines. For rescue of *egl-2* mutants, pTG44 and pTG46, which contain the *egl-2* cDNA expressed from the *unc-103E* and *lev-11* promoters, respectively, were injected at ng/µl along with 20ng/µl of pBL66. F1 hermaphrodite progeny with CFP expression in their intestines were selected. For each injection, 3-5 lines were analyzed; one representative line is shown in the tables.

To visualize PLC-3 expression, pTG92 (50ng/µl) was injected along with pBX1(100ng/µl) into *pha-1* hermaphrodites. 8 independent transmitting lines were analyzed; one representative is shown in the figure.

Laser ablations

We used standard laser ablation protocols to kill the pair of NSM neurons in the male pharynx [78]. We operated on L3 stage males and also placed non-operated L3 males on a pad soaked with anesthetic to rule out any effects it may have on behavior.

The locations of the NSMs were verified by the pTG11 *Ptph-1::*CFP reporter construct. The locations of the AWCs were verified by the pTG76 *Podr-3:*YFP reporter construct.

To assay the effects of NSM ablation on the Prc phenotype and pharyngeal pumping rate, we allowed animals from each experiment to recover for 2 days on NGM plates seeded with OP50 and then scored them for the phenotype. We measured effects on pharyngeal pumping by counting the number of posterior bulb contractions in one minute using a Zeiss Stemi SV11 microscope.

The *odr-3* promoter driving YFP was used to identify the AWC neurons in *unc-103(n1213) pha-1(e2123)* males. Ablations were originally done at L4 stage, however no effect was seen, and the *Podr-3:YFP* construct still expressed in the AWC neurons even in the adult animal, suggesting that ablation in L4 males was too late. However, when ablations were done in L3 stage, YFP expression disappeared in the AWC neurons in adult males. To rule out any effects of the transgene or *pha-1(e2123)*, ablations of AWC were then done in L3-stage *unc-103(n1213)* males and allowed to recover at 15°C overnight. The next day, males were moved to assay plates (food, inedible food, or no food) at late L4 larval stage and then scored for spontaneous spicule protraction 15-20hrs later. Males that dried up and died on the side of the plates were not scored.

LGG analysis

Autophagy is induced when cells cannot gain enough nutrients from the extracellular environment and results from intracellular protein breakdown by the

lysosome. Under these conditions, LGG-1 expression is punctate, whereas under well nourished conditions, the expression of LGG-1 is diffuse [79-81]. To visulalize autophagy in the head and body-wall muscles, we injected pTG90 (*Plev-11:LGG-1::YFP)* at 10ng/µl along with pBX1 (100ng/µl) and pUC18 (90ng/µl) into *pha-1* hermaphrodites. 5 independent transgenic lines were obtained and analyzed. To quantify the effects of food deprivation on autophagy levels, we visualized the dorsal and ventral head muscles for punctate expression in fed and 15 hour or 3 day food-deprived males. Males were picked as L4, and then placed on one of three conditions, NGM plates with *E. coli*, with aztreonam-treated *E. coli*, or without *E. coli*. Males were then visualized using fluorescence microscopy. LGG-1 puncta were not visualized after our standard 15-hour deprivation assay. However, puncta began emerging as starved animals grew older (3 days).

Nile Red staining

Nile Red staining was carried out as previously described [82]. Nile Red was added to plates previously seeded with OP50, and larvae on these plates were synchronized by hypochlorite treatment. L4-stage males stained with Nile Red (www.mpbio.com) were then added to one of the three different feeding plates (OP50, aztreonam-treated OP50, or no OP50), then visualized for Nile Red staining 15 hours later using a fluorescent microscope (100X). To quantify the number of fat droplets, equal planes of the animal were used and the region immediately posterior to the

pharynx was selected. Hermaphrodites were also visualized and showed a similar trend; however the data shown in the figure were obtained from males.

Analysis of Nile Red intensity changes were performed as described previously [82,83]. Briefly, images were captured by focusing on the first two intestinal cells. Nile Red intensity density was then quantified using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/). A region of interest was drawn around the first two intestinal cells and a 1.0 pixel Gaussian filter was used to identify fluorescent lipid droplets. The Analyze Particles function in ImageJ was used to count the number of lipid droplets, and generate a mask. The mask was then overlayed to the original ROI marking the first two intestinal cells, resulting in measurement of fluorescence in only in lipid droplets. Intensity Density (area × mean) of fluorescence specifically in lipid droplets is reported.

Calcium imaging with G-CaMP

pLR136 (*Punc-103E*:G-CaMP) was injected at 12ng/µl and pLR135 (*Punc-103E*:ds-Red) at 2ng/µl into *pha-1(2123)*; *lite-1(e314)* hermaphrodites (The *lite-1(ce314)* strain was graciously provided by Dr. Ken Miller, Oklahoma Medical Research Foundation). Ten independent transmitting lines were obtained and two lines with low to moderate background G-CaMP fluorescence were further analyzed. L4 males were separated from hermaphrodites the night before and allowed to mature into adults overnight. Worms were placed on a pad containing 2% Noble Agar dissolved in sterile

water, using a mouth pipette filled with S-Medium. The agar slide containing the worm was then placed on a cold block (~1°C) for no longer than 15 seconds. This immobilized the male long enough to glue him down. A borosilicate capillary tube was pulled and broken, leaving an opening at the tip about .16mm in diameter. This tube was used to apply a small amount of glue (Nexaband S/C) to the male via mouth pipette near the dorsal posterior end of the animal. A drop of S-Medium was placed on top of the worm and then covered with a cover slip. The dorsal protractor muscles and anal depressor muscle were focused on under normal light microscopy and then the focus and exposure time were refined using fluorescence. Animals were then recorded for intensity changes using Image Pro Plus Version 6.2 software (Media Cybernetics). Before dug application, recording of the image stream was started on the software and a LED flash light was used to mark the beginning of drug application (5-10s after recording begins), and the drug was applied by pipetting 50µl of drug the side of the coverslip. The drug then diffused across the pad and any intensity changes were recorded with Image Pro Plus. Fluorescence records were plotted as $\Delta F/F_0$, where F_0 was the average baseline value of fluorescence before any drug stimulus (i.e. before LED light flash in image sequence). The sex muscles were marked as a region of interest using Image Pro Plus software, and the percent change in fluorescence intensity for this region was plotted for each time point relative to F_0.

To measure G-CaMP intensity changes in the AWC neurons, pTG81 was injected into *pha-1(2123)*; *lite-1(ce314)* hermaphrodites at 35ng/µl. Six independent transmitting lines were obtained and one line with low background G-CaMP expression

was used for analysis. Well-fed, virgin, adult males were washed with M9 buffer, and placed on a 1.5% agar pad containing no bacteria or sodium azide. Vacuum grease was then placed around the circumference of the pad followed by a cover-slip to seal in the moisture. Each male was labeled and intensity measurements were then recorded from the left AWC neuron at each time point of starvation at 100x. After starvation, males were removed from the agar plates, placed on NGM plates seeded with OP50 and allowed to re-feed for 15hrs. Males were then mounted again on the agar pads and the G-CaMP intensity in the left AWC neuron was recorded. At each time point, the fluorescence of the left AWC neuron and the background fluorescence of the isthmus of the pharynx were recorded. Intensity measurements were then plotted as the percent intensity change over time relative to the background fluorescence.

Feeding GFP-expressing bacteria

To determine the edibility of aztreonam-treated *E. coli*, we used a CV2 strain of *E. coli* which constitutively expresses a GFP-containing plasmid (graciously provided by Gus Wright and Michael Manson, Texas A&M University). To test the edibility of aztreonam-treated *E. coli*, we treated this strain with aztreonam as described earlier and placed males on both treated and un-treated GFP-expressing bacteria. We found that we could visualize GFP-bacteria in the isthmus and grinder of the pharynx and in the intestines of males placed on the untreated bacteria. However, we never saw GFP bacteria in the intestines of aztreonam-treated GFP-bacteria, though in about 50% of the

animals, what appeared to be a long-chain of bacteria was seen trapped in the isthmus of the pharynx.

CHAPTER III

INTEGRATION OF MALE MATING AND FEEDING BEHAVIORS IN *C. elegans**

Male mating behaviors are suppressed by food deprivation

During the course of setting up genetic crosses with *C. elegans* males and hermaphrodites, we noticed that food-deprived males were less effective at mating than well-fed males. To quantify this observation, we measured the effect of 15-hr food deprivation on male-mating success (Figure 4A). We paired both well-fed and food-deprived virgin males with hermaphrodites and recorded the number of males that sired at least one progeny, given a 2-hr mating interval. Approximately 55% of well-fed males could sire at least one progeny, while only 20% of food-deprived males were successful ($p=0.001$, Fisher's exact test). The reduced mating success observed in food-deprived males could be the result of multiple mechanisms, involving many of the structures in the male genitalia. Here, we analyzed the spicule insertion step of mating, since inefficiency at this step is a likely reason for reduced success, and changes in sex-muscle output can be easily measured by observing spicule protraction.

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We hypothesized that one mechanism for food-deprivation suppression of mating may be through reducing the excitability of the sex muscles. To determine if food deprivation results in reduced muscle excitability, we tested sex-muscle response to a pharmacological acetylcholine (ACh) agonist previously shown to induce spicule protraction. The ACh agonist, arecoline (ARE), activates spicule protraction via Ca^{2+} influx from the L-type voltage-gated Ca^{2+} channel, EGL-19, which regulates tonic muscle contraction during mating. Similar to mating success, food deprivation reduced wild-type response to ARE-induced spicule protraction (Figure 4B). Specifically, we found that the effective concentration at which 50% of food-deprived males protracted their spicules (EC50) was 262µM, while the EC50 of well-fed males was 21µM.

In addition to measuring spicule protraction as a read-out for sex-muscle excitability, we also used the fluorescent calcium indicator, G-CaMP [73], to measure real-time calcium changes in the muscles (Figure 4C-D). G-CaMP consists of a circularly permutated enhanced-GFP connected to the M13 fragment of myosin light chain kinase and calmodulin (CaM).

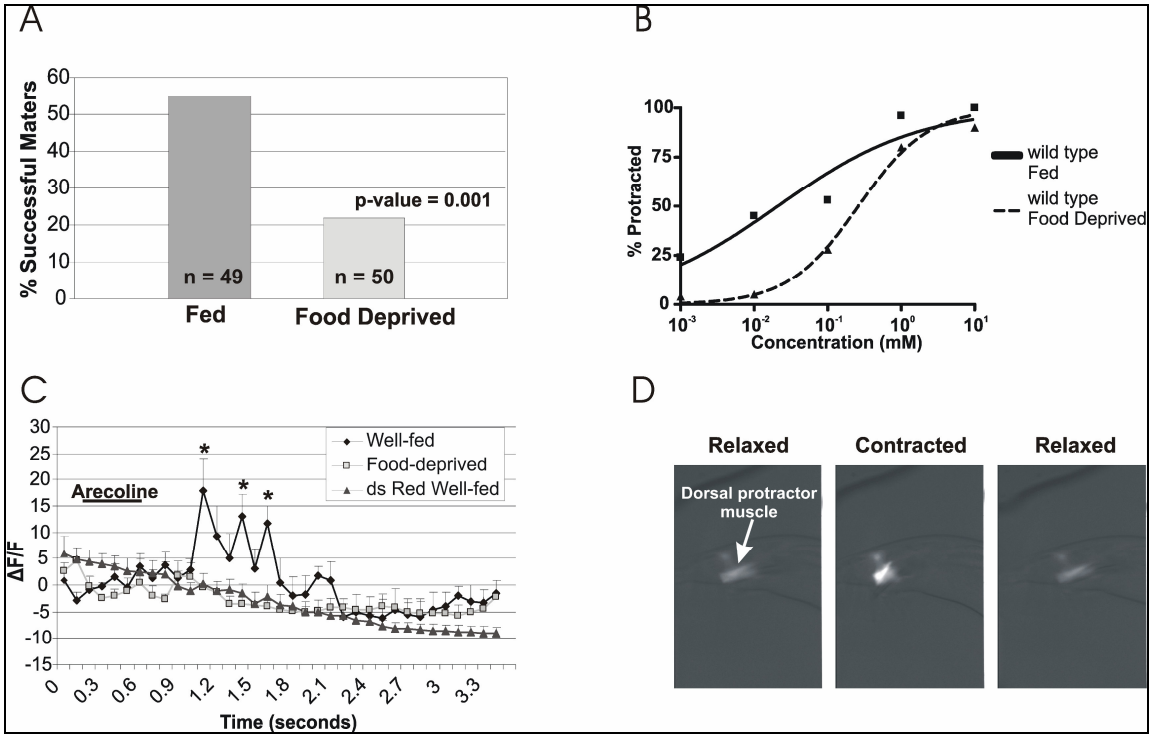

Figure 4. Effects of food deprivation on sex-muscle excitability.
(A) Mating success for fed and 15hr food-deprived males. Males were scored as successful if they sired at least 1 progeny. p-value determined by Fisher's exact test.
(B) Graph of male muscle arecoline (ARE) sensitivity. For each concentration assayed, 20-30 males were assayed.
(C) Mean sex-muscle G-CaMP responses to 10mM ARE for fed (n=4) and food-deprived (n=4) males. The * denotes well-fed time points that are significantly different ($p \leq 0.05$, Bonferroni posttest) then the food-deprived control. For fed males, the mean dsRed intensity trace is shown as a control. Error bars represent the standard error of the mean.
(D) Three representative frames displaying ARE-induced calcium changes in the sex muscles of a well-fed male (time between each frame is approximately 0.7 seconds). Anterior is to the right and the dorsal protractor muscles are labeled. Scale bar 6µm.

When calcium binds to CaM of G-CaMP, CaM binds M13 and the induced conformational change results in GFP intensity changes [73]. We hypothesized that reduced spicule protraction in response to ARE corresponded to decreased calcium signaling in the sex muscles of food-deprived males. To drive expression of G-CaMP in the sex muscles, we used the *unc-1031E* promoter (see *unc-103* expression pattern and site of action, p.42) [74]. Additionally, we also co-injected dsRed as a control for intensity changes caused by reduced cell volume or unintentional background changes. As predicted, we found that the spicule protractor muscles of food-deprived males had significantly lower calcium responses to ARE (Figure 4C). Specifically, well-fed males showed significant, rhythmic increases in fluorescence over time, while food-deprived males showed no increases. Taken together with the previous observations, these results suggest that food deprivation affects male mating by reducing the excitability of the sex muscles.

Food-deprivation assays do not cause muscle degradation or autophagy

Our results suggest that sex-muscle excitability is reduced under food-deprived conditions; however, it is still possible that reduced output could be the result of muscle degradation or autophagy. Autophagy, or the catabolism of cellular components, increases during nutrient deprivation and results in intracellular protein breakdown by the lysosome [84]. To test this directly, we measured autophagy levels using a GFP-tagged LGG-1, which encodes the *C. elegans* ortholog of yeast Apg8/Aut7p and

mammalian MAP-LC3. LGG-1 can be used to mark preautophagosomal and autophagosomal membranes; under nutrient-deprived conditions, the expression of LGG-1 is punctate, whereas under well-nourished conditions, its expression is diffuse [79-81].

We used a body-wall and sex-muscle specific promoter (*Plev-11)* to drive a GFP-tagged LGG-1, and measured the levels of autophagy in both well-fed and 15hr food-deprived conditions. Interestingly, we found no significant increases in autophagy under food-deprived conditions. Both well-fed and food-deprived males displayed a diffuse expression pattern of LGG-1::GFP (Figure 5A-B). However, LGG-1::GFP puncta began emerging as animals were deprived for longer intervals (3 days) (Figure 5B). These results suggest that the food-deprivation intervals (15hrs) in our assays are not severe enough to induce autophagy or muscle breakdown, and that food deprivation suppresses muscle excitability via other mechanisms.

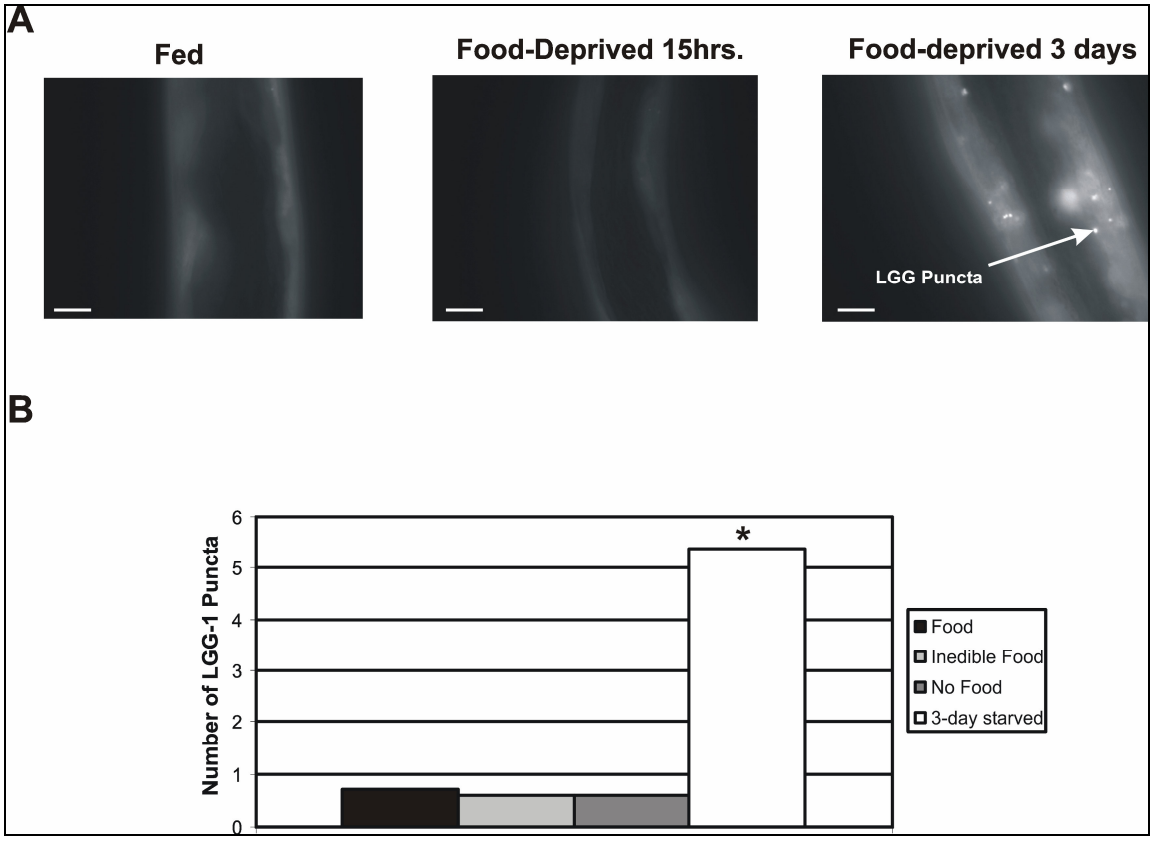

Figure 5: Food deprivation does not induce muscle degradation.
(A) Representative pictures of males expressing LGG-1 in 3 separate feeding conditions, Fed, Food Deprived for 15hrs, and Food Deprived for 3 days (scale bar 9µm).
(B) Graph displaying the number of LGG-1 puncta in males under four different conditions, Fed, Inedible Food 15hrs, No Food 15hrs, and starved for 3-days. For each condition, 10-20 males were analyzed. The * indicates a significant difference ($p<0.001$) then the other 3 feeding conditions.

Spicule protraction mutants vary in response to environmental conditions

To understand how *C. elegans* males regulate their spicule muscles, a previous screen isolated mutations that cause males to suffer spontaneous sex-muscle seizures and protract their spicules [56]. This screen identified the ERG-like K^+ channel/UNC-103, as a key component required to suppress sex-muscle output during periods between mating [46]. While working with spicule protraction mutants, we noticed that the percentage of males displaying the seizure phenotype varied with temperature, population size, amount of moisture, and availability of food. To determine more quantitatively if nutritional or environmental perturbations could affect the spicule mutant phenotype, we compared the effects of growth in liquid and in the presence or absence of food (Table 1). *unc-43(sy574)*, which affects calcium/calmodulin-dependent kinase II (CaMKII), and *egl-19(n2368gf)*, which affects the L-type voltage-gated Ca^{2+} channel (L-VGCC), also cause spontaneous sex-muscle seizures and were used to determine if feeding state or liquid growth affected all seizure mutants similarly.

To measure the effects of food and liquid growth on spicule protraction, we removed L4 larval stage males from plates with food and reared them for 24h in buffer with or without food (*E. coli*, OP50). Interestingly, we found that the absence of food during the last larval molt can suppress *unc-103(0)*-induced seizures, but had no effect on *unc-43(sy574)* or *egl-19(n2368gf)*. Additionally, we found that rearing *unc-103(dn)* in liquid culture partially suppressed the protraction defect, possibly masking any effects mediated by the absence of food (Table 1). To test this more directly, we also analyzed

the effects of food deprivation alone by placing males on agar plates without bacteria. We found that depriving both *unc-103(0)* and *unc-103(dn)* of food resulted in a significant reduction in males who suffer spontaneous muscle seizures from 35% to 9% and from 79% to 11%, respectively (Table 1). These results suggest that there are compensating mechanisms activated in response to liquid growth and food deprivation that can reduce cell-excitability, and consequently restore control of seizures in *unc-103(lf)* mutants.

Table 1. Effects of food availability on spicule protraction mutants

Genotype	Percent Spicule-Protracted Males (n)			
	NGM Plates + *E. coli*	S-medium + *E. coli*	S-medium	NGM Plates
Wild type	9 (66)	0 (20)	0 (20)	0 (26)
unc-103(0)	35 (20)	37 (30)	10 (20)[b]	9 (23)[a]
unc-103(dn)	79 (61)	47 (32)[a]	34 (32)	11 (35)[a]
unc-43(sy574)	46 (24)	43 (28)	52 (27)	50 (44)
egl-19(n2368gf)	100 (10)	100 (10)	100 (10)	100 (10)

[a]p-value<0.05 compared with plates plus food, χ^2 test

[b]p-value<0.05 compared to S-medium plus food, χ^2 test

Although how these environmental conditions are suppressing *unc-103(lf)*-muscle seizures is not clear, the effect appears to be a specific, regulated response rather than a general suppression of muscle function. This is supported by the observation that neither liquid growth nor food deprivation suppressed sex-muscle seizures in *unc-43(sy574lf)* or *egl-19(n2368gf)* mutant males (Table 1). Additionally, CaMKII and L-type voltage-gated Ca^{2+} channels may be molecular components of the compensating mechanisms activated by food deprivation and liquid growth.

unc-103 expression pattern and site of action

To determine how food deprivation could be acting to suppress *unc-103(lf)*-induced sex-muscle seizures, we first determined the expression pattern and site of action of *unc-103*. Previous studies reported that an *unc-103::*GFP translational fusion containing sequences 5kb upstream of the *unc-103* start codon expresses in the SPC and PCB cholinergic motor neurons that regulate spicule muscle contraction. This translational fusion also expressed in the AS ventral cord neurons, as well as various head neurons (ALA, ADL, ASK, AVH, AVJ, AIN, AVA, ASJ, SMDD, SIA, ADE, and AVD) [56]. To study the expression pattern in more detail, we made an expression construct using a larger promoter region (8.7kb upstream) to drive YFP (Figure 6A-D). We found that *unc-103* also expresses in pharyngeal neurons (I2 and NSM) (Figure 6B), head neurons (Il1, IL2, OLL, URAD, ASH, AVD, AUA, and SIAV) (Figure 6B), and OLQ, RIV, URYV, AIN, and AIA (data not shown), PCA in the post cloacal sensilla, and one of two ray neurons in rays 1,2,3,4,6, and 9 (data not shown). Additionally, we also injected a *unc-103::*YFP translation fusion expressed from the same 8.7kb promoter region and observed that the anal depressor, spicule protractors, spicule retractors, and other sex muscles also expressed *unc-103* (Figure 6D). These results suggested that *unc-103* is expressed in multiple tissues, and could be acting in multiple tissues to control spicule protraction.

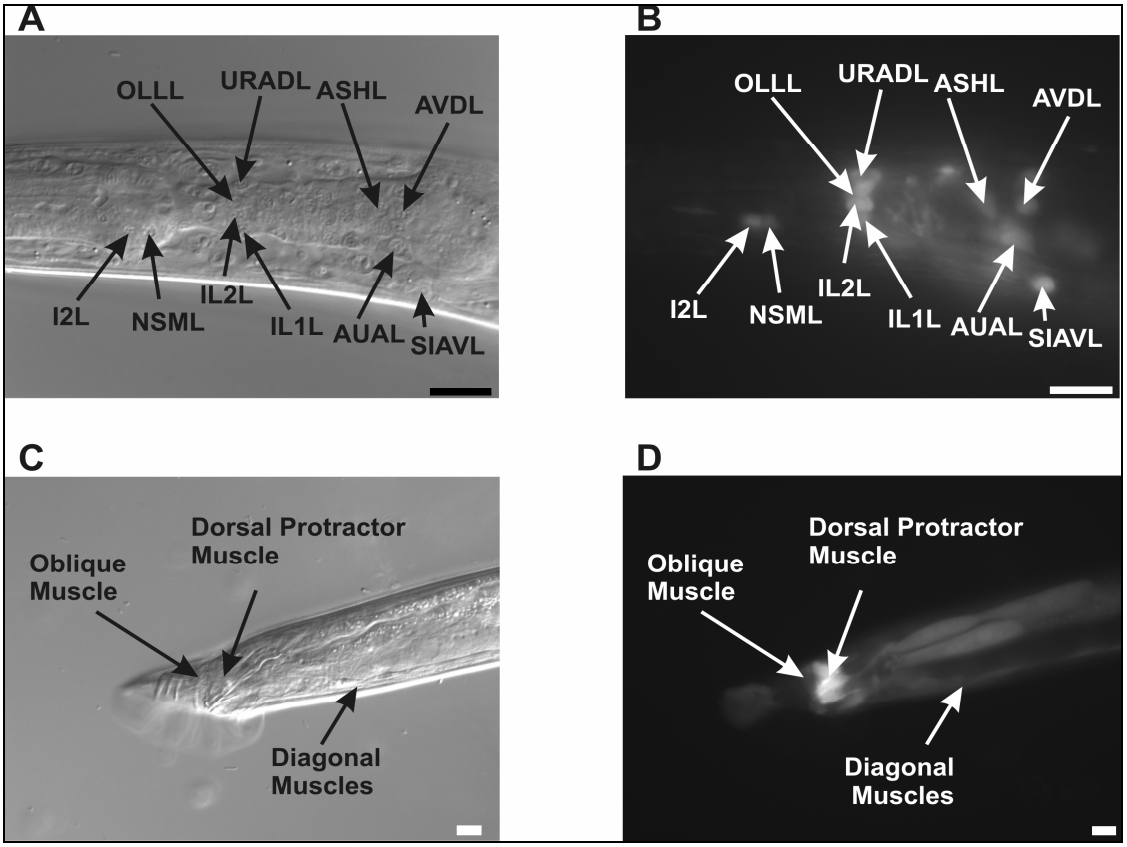

Figure 6: *unc-103* expression pattern in the *C. elegans* male.
(A-B) A, Nomarski and B, fluorescence images of the left lateral head region of a male expressing the *unc-103*::YFP promoter construct (pLR10). Pharyngeal and head neurons in the focal plane are labeled; scale bar, 10 μm.
(C-D) C, Nomarski and D, fluorescence images of the right lateral tail region of a male expressing the *unc-103*::YFP translational fusion. Sex muscles in the focal plane are labeled; scale bar, 10 μm.

To characterize the functional coding and promoter sequences of *unc-103*, we used 5' and 3' RACE. We found potentially 18 different *unc-103* isoforms, including six alternative first exons that are spliced to exon 2. This suggested that separate promoters may drive expression of the different first exons. Therefore, YFP reporters were constructed containing the upstream sequences for each of the six first exons [74]. Each

first exon had its own specific expression pattern, including sex-muscle specific and sex-neuronal-specific isoforms. The size of the upstream sequences for each of the six alternative first exons and their respective expression pattern are listed in the appendix (Appendix A, Table A-1).

The expression pattern of *unc-103* indicates that it is expressed in both the neurons and the muscles that regulate spicule protraction. Therefore, we asked which of these cells require *unc-103* function to suppress spontaneous muscle seizures on standard-food conditions. We used promoter regions of the six alternative first exons to generate tissue- and isoform-specific rescue constructs and injected them into *unc-103(0)* hermaphrodites. We found that the neuronal-specific *Punc-103F* promoter driving the *unc-103F* isoform did not rescue *unc-103(0)*-induced spicule protraction. However, the *unc-103F* and *unc-103E* isoforms driven by the sex-muscle specific promoter, *Punc-1031E*, rescued *unc-103(0)*-induced seizures (Table 2). Additionally, the *unc-103E* isoform driven by the sex-muscle *Punc-103E* promoter also rescued *unc-103(dn)*-muscle seizures. Despite the broad expression pattern of *unc-103*, these results suggest that *unc-103(lf)* mutations specifically affect the sex muscles to induce spontaneous seizures. We therefore concluded that this phenotype can be used as a proxy to measure how different environmental conditions influence sex-muscle excitability.

Table 2. Transgenic rescue of mutant ERG K+ channel/UNC-103-induced spicule protraction			
Genotype[a]	Tissue Expression	% Spicule-Protracted Males (n)	p-value[b]
unc-103(0)		39 (67)	
unc-103(dn)		81 (21)	
unc-103(0); rgEx74[P aex-3 ::unc-103F(+)]	Panneuronal expression	29 (53)	
unc-103(0); rgEx78[P unc-103F ::unc-103F(+)]	Specific neuronal expression	45 (40)	
unc-103(0); rgEx76[P lev-11 ::unc-103F(+)]	Body-wall, anal, and sex-muscle specific	3 (30)	<0.005
unc-103(0); rgEx79[P unc-103E ::unc-103F(+)]	Spicule proractor muscles + and few head neurons	2 (44)	<0.005
unc-103(0); rgEx81[P unc-103E ::unc-103E(+)]	Spicule proractor muscles + and few head neurons	2 (46)	< 0.005
unc-103(dn); rgEx81[P unc-103E ::unc-103E(+)]	Spicule proractor muscles + and few head neurons	0 (22)	< 0.005

[a]All strains contain *pha-1(ts); him-5*
[b]Based on Fisher's exact test

Chapter summary

In Chapter III, I demonstrated that food deprivation reduces male-mating success, and that one possible mechanism for this effect is reduction in genital-muscle excitability. Food deprivation results in reduced sex-muscle output in response to pharmaceutical ACh agonists, corresponding to decreased Ca^{2+} signaling as measured by the fluorescent Ca^{2+} indicator, G-CaMP. This reduction in muscle excitability cannot be explained by increased muscle degradation, since food-deprivation periods were not severe enough to induce autophagy as measured by an LGG-1::GFP marker. Instead, food deprivation appears to activate mechanisms that can suppress membrane excitability of the sex muscles. This is supported by the finding that in addition to suppressing response to various drug-induced contractions, food deprivation also suppresses spontaneous sex-muscle seizures caused by loss of ERG-like/UNC-103 K^+

channels. Since UNC-103 acts in the sex muscles to suppress spontaneous muscle output, we will use the *unc-103(lf)*-seizure phenotype to dissect how food deprivation influences sex-muscle excitability.

CHAPTER IV

PHARYNGEAL ACTIVITY AND SENSORY PERCEPTION OF FOOD REGULATE SEX-MUSCLE EXCITABILITY*

Tropomyosin (*lev-11*) mutants suppress *unc-103(lf)*-induced seizures

Since food deprivation can suppress *unc-103(lf)*-induced seizures, we reasoned that genetic suppressors of the *unc-103(lf)* would identify potential molecules that act during food deprivation to suppress sex-muscle excitability. A suppressor screen in the *unc-103(dn)* background identified the *rg1* allele of tropomyosin (*lev-11)*, which suppresses *unc-103(dn)* from 82% to 17% and *unc-103(0)* from 42% to 1%. This allele is semi-dominant and can suppress the *unc-103(dn)* allele from 82% to 42% when heterozygous (χ^2 test; $p < 0.05$).

lev-11 encodes the worm homolog of tropomyosin, a thin-filament muscle protein that regulates Ca^{2+} mediated muscle contraction [76,85-87]. The *lev-11* gene in *C. elegans* contains at least four tropomyosin isoforms that are expressed in different muscle types [76,88]. It was previously reported that the four known isoforms can be classified into two groups: CeTMI and CeTMII express in the body-wall, anal, and sex

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*Portions of this chapter are reprinted with permission from Gruninger TR, Gualberto DG, LeBoeuf B, Garcia LR (2006) Integration of male mating and feeding behaviors in *Caenorhabditis elegans*. J Neurosci 26: 169-179 and from Gruninger TR, Gualberto, DG, Garcia LR (2008) Sensory perception of food and insulin-like signals influence seizure susceptibility. PLoS Genetics: in Press.

muscles from a promoter region located 600-800bp upstream of the start codon, and CeTMIII and CeTMIV express in the muscular feeding organ called the pharynx and in the intestine from an internal promoter located within the third intron (Figure 7A). *lev-11(rg1)* causes a E222K change in exon 7 of *lev-11*, near one of the proposed sites of regulation (troponin T (TNT) binding site). Because exon 7 is found in both *lev-11* tissue types, this substitution should affect all muscle types (Figure 7A).

lev-11(rg1) disrupts multiple steps of male mating, except for spicule insertion

Since tropomyosin is a thin filament protein involved in Ca^{2+} regulation of muscle contraction, we initially hypothesized that the altered tropomyosin in *lev-11(rg1)* mutants may cause general weak sex-muscle functions. However, we accumulated multiple observations that contradict this hypothesis. First, *lev-11(rg1)* does not suppress the spontaneous spicule protraction caused by mutations in L-VGCC/*egl-19* (*egl-19(n2368gf)*) or CaMKII/*unc-43(sy574)*. About 98% of *lev-11(rg1); egl-19(n2368gf)* males suffer spontaneous seizures compared to 100% displayed by *egl-19(gf)* single mutants, while 56% of *lev-11(rg1); unc-43(sy574)* males suffer spontaneous seizures compared to 48% displayed by *unc-43(sy574)* single mutants. This suggests that there is some specificity to how the altered tropomyosin acts with the different seizure mutations (Table 3). In contrast to the *unc-103(lf)* alleles, the phenotypes of *egl-19(n2368gf)* and *unc-43(sy574)* are also not suppressed by food deprivation, suggesting that *lev-11(rg1)* may identify a specific pathway used when the male encounters a low-food environment.

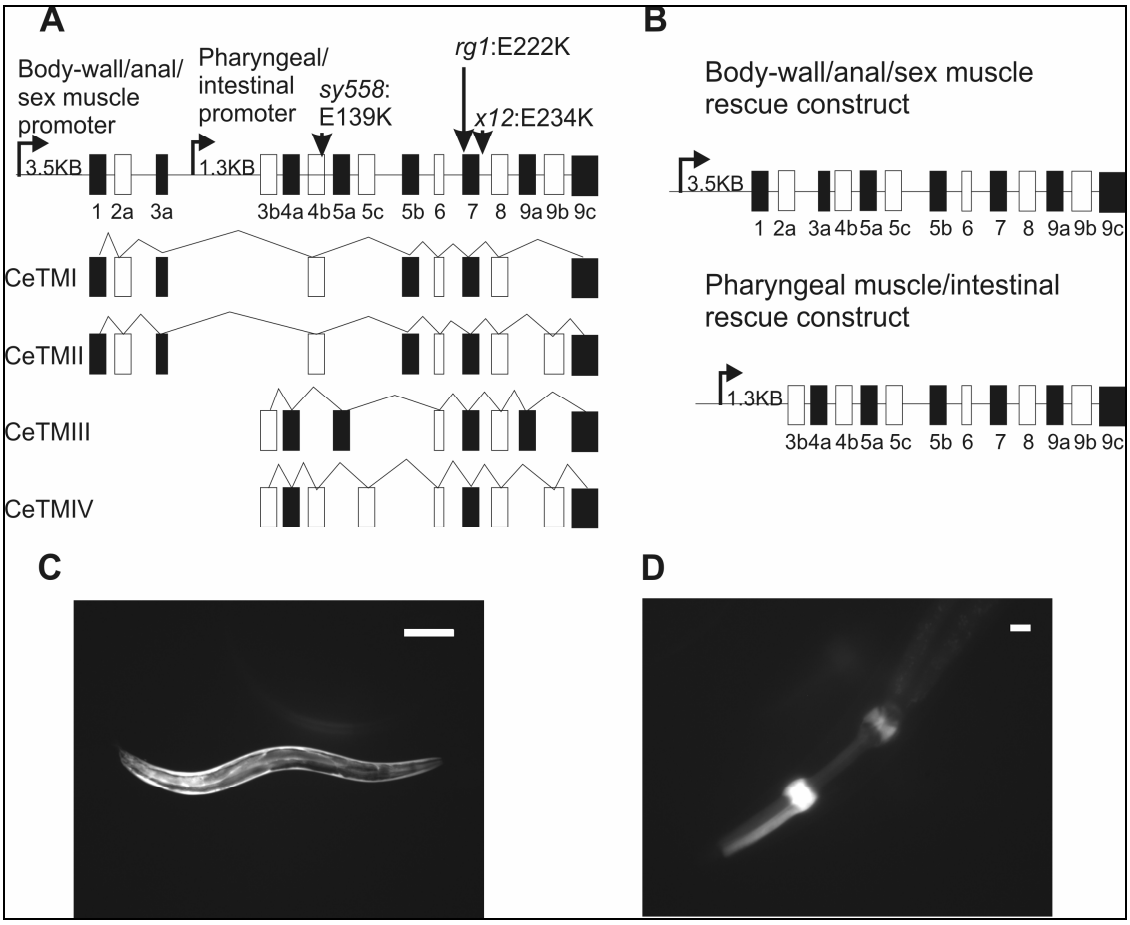

Figure 7. The *lev-11* gene contains two groups of tissue-specific isoforms.
(A) The *lev-11* genomic structure, highlighting the previously reported tissue-specific promoters (adapted from Anyanful et al 2001). The locations of the *sy558*, *rg1*, and *x12* alleles are shown with arrows.
(B) *lev-11* sequences used to build tissue specific rescue constructs.
(C) Expression pattern of the promoter sequences that drive *lev-11* in body-wall/anal/sex muscles; scale bar, 100µm.
(D) Expression pattern of promoter sequences that drive *lev-11* in the pharyngeal muscle and intestine; scale bar, 10 µm.

lev-11(rg1), as with other alleles of *lev-11*, results in body-wall muscle resistance to the ACh agonist, levamisole, which is the origin of the gene name [59]. However, *lev-11(rg1)* only slightly reduces sex-muscle sensitivity to levamisole by a factor of two [60]. Taken together with the observation that *lev-11(rg1)* does not suppress all spicule protraction mutants, we concluded that the small levamisole insensitivity of *rg1* spicule protractor muscles could not sufficiently explain suppression of *unc-103(lf)*-induced sex-muscle seizures.

Since our observations suggested that *lev-11(rg1)* affects multiple muscle types, yet does not simply result in nonspecific weak sex-muscle function, we observed the mating ability of mutant males. To quantify the mating potency of *lev-11(rg1)* males, we paired single males with paralyzed hermaphrodites. From our assay, we found that 69% of wild-type males (n=48) can sire at least one progeny, while 31% of *lev-11(rg1)* males (n=52) can sire at least one progeny. To determine if reduced mating potency could reveal how mutant tropomyosin can suppress *unc-103(lf)* induced seizures, we observed the mating behavior of *lev-11(rg1)* and *lev-11(rg1); unc-103(dn)* males in more detail. Interestingly, we found that the *lev-11(rg1)* allele disrupts most steps of male mating, except for the spicule insertion step. We observed that 78% of wild-type males will immediately initiate mating behavior within three tail contacts with a hermaphrodite, while only 33% of *lev-11(rg1)* males respond to tail contact (Table 4). In addition, 73% *lev-11(rg1)* males are unable to complete an efficient turn upon reaching the end of the hermaphrodite, and 60% of the males cannot locate the hermaphrodite vulva within the first three encounters (Table 4). Interestingly, when *lev-11(rg1)* males successfully

recognize the vulva and attempt spicule insertion, they contract their spicule muscles at a similar rate as wild-type males, 132 ± 60s (n=8) for *lev-11(rg1)* compared to 122 ± 107s (n=9) for wild type. These results suggest that *rg1* disrupts the steps that occur before spicule insertion. In contrast to *lev-11(rg1)* single mutants, we found that *lev-11(rg1)*; *unc-103(dn)* double mutants were partially rescued for *lev-11(rg1)* mating defects (Table 4). Therefore, in addition to its role in regulating sex-muscle excitability, UNC-103 may subtly regulate other behavioral steps in male mating.

Table 3: Tropomyosin/*lev-11* mutations suppress *unc-103(lf)*-induced spicule protraction

Genotype	% Males Protracted	*n*
Wild type	4	26
unc-103(dn)	82	43
unc-103(0)	42	91
egl-19(tg26gf)	100	30
unc-43(sy574)	48	79
lev-11(rg1)	0	26
lev-11(x12)	0	20
lev-11(rg1); unc-103(dn)	17	126
lev-11(rg1)/+; unc-103(dn)	42	41
lev-11(x12); unc-103(dn)	13	72
lev-11(rg1); unc-103(0)	1	69
lev-11(rg1); egl-19(tg26gf)	98	57
lev-11(rg1); unc-43(sy574)	56	54

The turning defect seen with *lev-11(rg1)* suggests that this lesion is affecting the specialized muscle cells called the diagonal muscles, which are required to complete the movements necessary for turning behavior [44]. Similarly, the tail contact defect and

vulva location defect of *lev-11(rg1)* males suggest that the *rg1* lesion is specifically affecting sensory-motor responses. This led us to the hypothesis that although *lev-11(rg1)* may slightly reduce spicule muscle function, it may also affect other mechanisms that repress specific sensory-motor responses required during mating behavior.

Pharyngeal-specific and body-wall-specific LEV-11 regulate the sex muscles

lev-11(rg1) could be affecting multiple tissues to suppress *unc-103(lf)*-induced seizures, since it is located in an exon contained in all tropomyosin isoforms. Even though *lev-11(rg1)* is a semidominant allele (Table 3), *lev-11(+)* genomic DNA injected into *lev-11(rg1)*; *unc-103(dn)* can partially restore the *unc-103*-mediated seizure phenotype from 12% (n=25) to 57% (n=30; χ^2 test; $p \leq 0.05$). Therefore, we generated tissue-specific rescue constructs of *lev-11* to determine what muscle type the *rg1* lesion affects to suppress *unc-103(lf)* seizures. To drive expression of *lev-11(+)* DNA in the body-wall, anal, and sex muscles, we employed extension overlap PCR to amplify sequences 3.5kb upstream of exon 1 and only the specific exons found in CeTMI and CeTMII, thereby omitting the internal promoter and exons specific to CeTMIII and CeTMIV (Figure 7B). To drive expression of *lev-11(+)* in the pharyngeal muscles and intestine, the 1.3kb internal promoter and the rest of the genomic sequences were amplified. Each PCR product was then injected into *lev-11(rg1); unc-103(dn)* and *lev-11(rg1)*; *unc-103(0)* hermaphrodites. To verify the specificity of the promoters, we also

generated transcriptional fusions of the 3.5kb external promoter and the 1.3kb internal promoter to GFP (pDG9) and CFP (pTG13), respectively. The 3.5kb upstream region drove expression of GFP in the body-wall muscles, the anal depressor muscle, and the sex muscles; whereas the 1.3kb internal promoter drove expression of CFP exclusively in the pharyngeal muscle and intestine (Figure 7C-D).

Table 4: Both body-wall/anal/sex-muscle specific and pharyngeal/intestinal specific *lev-11* regulate spicule protraction

Relevant Genotype	% males protracted	% levamisol resistant	% abnormal turning	% abnormal vulva location	% abnormal response
Wild type	9 (23)[a]	0 (23)	13 (23)	13 (23)	22 (23)
unc-103(dn)[b]	85 (33)	n.d.	n.d.	n.d.	n.d.
unc-103(0)[b]	35 (45)	n.d.	n.d.	n.d.	n.d.
lev-11(rg1)	0 (15)	100 (10)	73 (15)	60 (15)	67 (15)
lev-11(rg1); unc-103(dn)[b]	17 (126)	100 (14)	47[d] (34)	38[d] (34)	18[d] (34)
lev-11(rg1); unc-103(0)	1(69)	100 (10)	50 (10)	40 (10)	50 (10)
lev-11(rg1); unc-103(dn) rgEx26(body wall/anal/sex muscle lev-11(+))[b]	35[c] (37)	39[c] (18)	9[c] (11)	45 (11)	0 (11)
lev-11(rg1); unc-103(dn); rgEx27(pharyngeal muscle/intestine lev-11(+))[b]	35[c] (46)	100 (11)	25 (12)	8[c] (12)	0 (12)
lev-11(rg1); unc-103(0) rgEx26(body wall/anal/sex muscle lev-11(+))[b]	0 (17)	25[c] (16)	n.d.	n.d.	n.d.
lev-11(rg1); unc-103(0) rgEx27(pharyngeal muscle/intestine lev-11(+))[b]	22[c] (41)	100 (10)	n.d.	n.d.	n.d.

[a] All numbers in parentheses refer to number of animals assayed
[b] Strains contain *pha-1(e2123)* and the *pha-1* rescuing plasmid, pBX1
[c] Chi-squared test p-value < 0.05 compared to *lev-11(rg1); unc-103(sy557)* or *lev-11(rg1); unc-103(n1213)*
[d] Chi-squared test p-value < 0.05 compared to *lev-11(rg1)*

Interestingly, we found that both the body-wall/anal/sex-muscle and pharyngeal/intestinal constructs restored *unc-103(dn)*-induced seizures (Table 4). However, when we injected these constructs into the *lev-11(rg1)*; *unc-103(0)* background, we found that only the pharyngeal construct restored the spontaneous protraction phenotype, increasing the number of males with spontaneous seizures from 1% to 22% (Table 4). Additionally, while the body-wall/anal/sex-muscle construct restored levamisole sensitivity and turning behavior, the pharyngeal/intestinal construct surprisingly restored the vulva location defect, increasing the percentage of males that could successfully recognize the vulva from 62% to 92%. These results suggest that the pharyngeal/intestine rescue construct can distinctly affect the tissues controlling vulva location and spicule protraction. We conclude that *lev-11(rg1)* must be suppressing *unc-103(dn)* by affecting the body-wall, anal, sex muscles, and the pharyngeal muscles, while *lev-11(rg1)* must be suppressing *unc-103(0)* mainly in the pharyngeal muscle and/or intestines. These results suggest that muscular activity in the pharynx and/or the intestines can somehow regulate sex-muscle output.

RNAi of pharyngeal-specific troponin T suppresses *unc-103(dn)*

Since our results indicate that two tissues involved in feeding and metabolism, the pharynx and intestines, can affect sex-muscle activity, we tried to phenocopy the *lev-11(rg1)-unc-103(dn)* interaction by looking at other tissue-specific molecules that

regulate muscle contraction. The *rg1* lesion is located near the proposed troponin T (TnT) binding site on tropomyosin, suggesting it may perturb interaction with this protein [76]. TnT forms a three-subunit complex along with troponin C and troponin I that mediates calcium regulation of muscle contraction [89]. In *C. elegans*, there are four separate TnT genes, and we asked which TnT, if any, could suppress *unc-103(dn)*-induced seizures when knocked down via RNAi. To verify that the effects of TnT knockdown were specific to *unc-103* mutants and not all spicule protraction mutants, we also assayed a mutant allele that is not suppressed by food-deprivation, *unc-43(sy574)*. Of the four TnTs analyzed, only RNAi of *tnt-4* significantly suppressed *unc-103(dn)* from 45% to 0% (Figure 8A). However, RNAi of *tnt-4* had no effect on *unc-43(sy574)*, demonstrating the specificity of *tnt-4* in *unc-103*-mediated spicule protraction. Additionally, reducing *tnt-1* and *tnt-3* can suppress *unc-43(sy574)*-induced seizures, suggesting that although they do not significantly affect *unc-103(dn)*, reducing their expression can suppress sex-muscle contractions caused by defects in other genes.

To identify the cells where *tnt-4* is expressed and could be acting to affect sex-muscle output, we constructed a GFP-translation fusion (pTG7) that contains sequences that spans from 3kb upstream of *tnt-4* to the last exon of the smaller *tnt-4* isoforms (www.wormbase.org). Interestingly, we found that this GFP reporter construct expressed only in the pharyngeal muscles (Figure 8B). Taken together with the *lev-11(rg1)* rescues, we propose that pharyngeal muscle physiology can regulate male sex-muscle excitability.

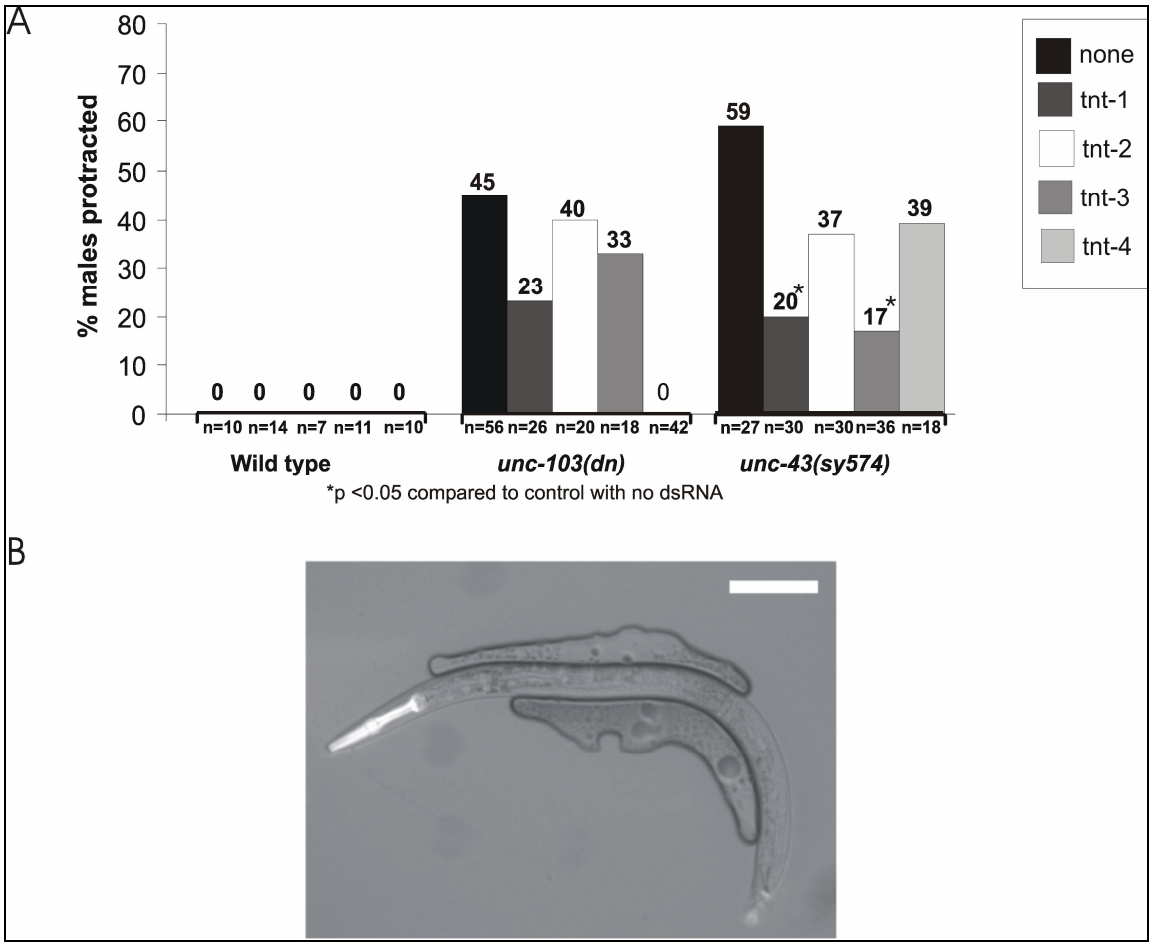

Figure 8. Reducing the pharungeal-specific troponin-T, TNT-4, via RNAi supresses *unc-103(lf)*-seizures.

(A) Specific troponin T proteins are required for different Prc mutants. The numbers in the columns indicate the percentage of males that display the Prc phenotype after being soaked in S-medium containing the corresponding dsRNA, all numbers in parentheses refer to number of animals assayed. The asterisk designates numbers significantly different ($p > 0.05$) compared to control with no dsRNA.

(B) Merged Nomarski and fluorescence image of an adult male that expresses the tnt-4::GFP reporter construct. Scale bar, 100µm.

Pharyngeal signals are transmitted via the neurosecretory (NSM) neurons

To determine how changes in the pharyngeal muscle could influence *unc-103(lf)*-induced sex-muscle seizures, we asked which neurons could possibly communicate between these two tissues. We reasoned that the pair of pharyngeal NSM neurons was a likely candidate. Specifically, the NSM neurons have proprioceptive-like endings with processes located in the lumen of the pharynx, where ingested bacteria could be sensed. They also contain processes that run close to the pseudocoelom, which contain varicosities filled with large, light- and dark-staining membrane-bound vesicles. These characteristics have led to the suggestion that the NSMs may be able to sense the ingestion of food through their sensory endings and then signal to the rest of the animal via the secretion of neuroactive molecules into the pseduocoelom [90]. Consequently, we asked if the NSMs are required for *lev-11(rg1)*-mediated suppression of *unc-103(dn)*.

We identified the NSM neurons using a *tph-1:*GFP reporter construct (pTG11). *tph-1* encodes tryptophan hydroxylase, an enzyme required for serotonin biosynthesis that is expressed in all serotonergic neurons, including the NSMs [72]. *lev-11(rg1); unc-103(dn)* and *lev-11(rg1); unc-103(0)* males were isolated at the L3 larval stage, and the NSM neurons were ablated using a laser microbeam. Killing the NSM neurons significantly restored spontaneous protraction in both genetic backgrounds: from 11% to 45% for *lev-11(rg1); unc-103(dn)* and from 7% to 39% for *lev-11(rg1); unc-103(0)* (Figure 9A). These results indicate that the NSMs can suppress sex-muscle excitability in response to pharyngeal changes.

Because our data indicates that altered pharyngeal muscle proteins can influence sex-muscle excitability via the NSM neurons, we asked if the *lev-11(rg1)* mutation significantly changes pharyngeal muscle-contraction rates. To test this, we measured pharyngeal contractions per minute for *lev-11* and *unc-103* single and double mutants (Figure 9B). *lev-11(rg1)* mutants had a significantly lower ($p<0.001$, Mann Whitney test) pharyngeal pumping rate than wild-type or *unc-103(dn)* males. Surprisingly, we found that the pumping defect was masked in *lev-11(rg1); unc-103(lf)* double-mutants.

Although previous studies have demonstrated that the NSMs are not required for normal pumping activity in well-fed animals, we hypothesized that the NSM neurons may coordinate pharyngeal muscle contractions and sex-muscle excitability under non-optimal conditions, such as when pharyngeal muscle activity is altered. To test this more directly, we ablated the NSM neurons in *lev-11-unc-103* single and double mutant backgrounds and assayed the effects on pharyngeal pumping rate (Figure 9B). Consistent with the previous data, we found no effect on pumping activity when we ablated the NSMs in wild type. However, we found that ablation of the NSM neurons significantly reduced pharyngeal pumping rate in *lev-11* and *unc-103* mutant backgrounds. NSM ablation reduced the pumping rates of *lev-11(rg1)* mutants from 161 to 95 pumps/minute, *unc-103(dn)* from 217 to 146 pumps/minute, and *unc-103(0)* from 186 to 146 pumps/minute. These results suggests that in intact animals, NSM activity can partially counteract the decreased pumping rate in caused by the *lev-11(rg1)* and *unc-103(lf)* mutations.

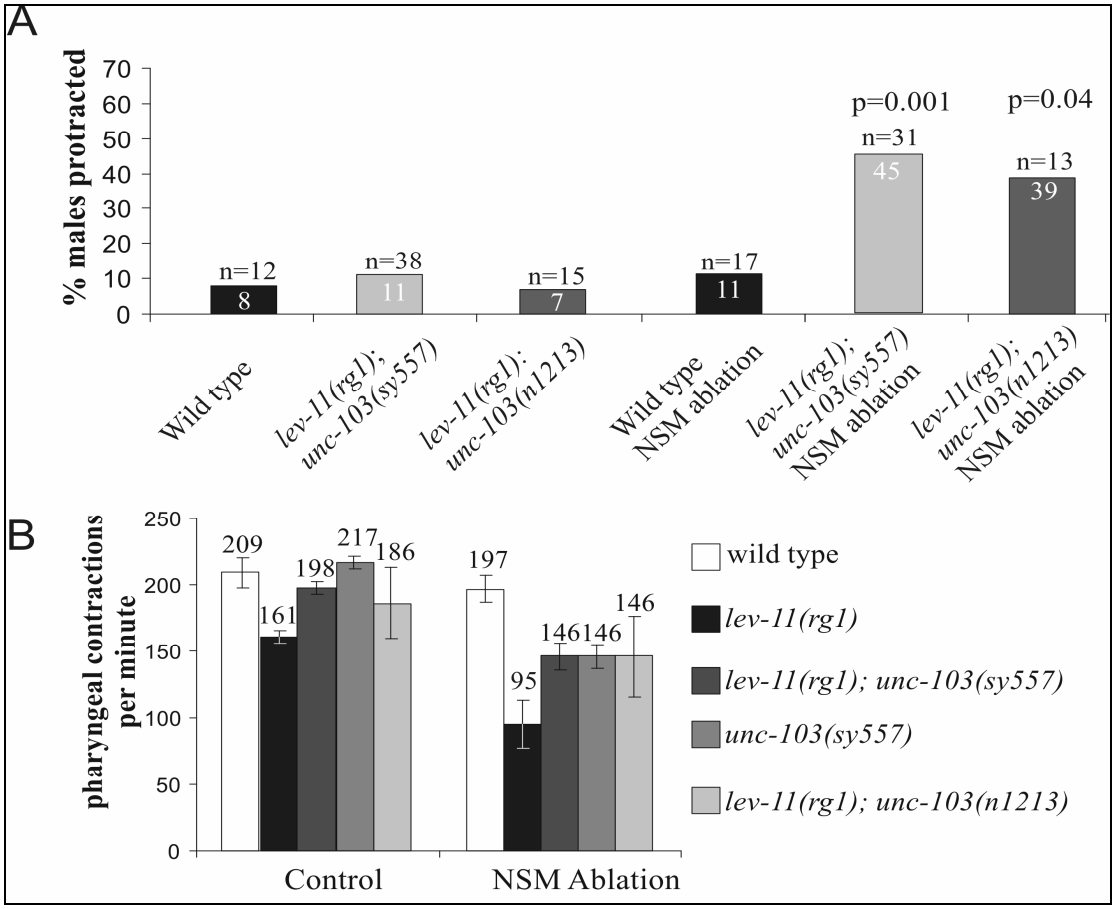

Figure 9. The pharyngeal NSM neurons coordinate pharyngeal and sex-muscle output.
(A) Effects of ablating the bilateral pair of NSM neurons on the Prc phenotype. p-values based on Chi-squared test.
(B) Effects on pharyngeal pumping rate when the NSMs are ablated. Error bars represent the standard error of the mean.

To investigate the function of the NSM neurons in more detail, we ablated the NSMs in wild-type males and measured the effects on mating behavior. Liu and Sternberg [45] had previously reported that pharyngeal pumping rate decreases and becomes erratic during male mating, and we hypothesized that this effect may be

mediated by the NSM neurons. We paired intact and NSM-ablated wild-type males with paralyzed hermaphodites and observed the effects on contact response, turning, vulva location, spicule insertion, and pumping rate during mating. NSM-ablated males showed no significant difference in any of the mating substeps (data not shown). However, pharyngeal pumping rate during mating was affected. Specifically, we found that 100% of wild-type males (n=11) reduced pharyngeal pumping rate after vulva contact, while only 55% of NSM-ablated males (n=11; χ^2 test; $p<0.05$) reduced their pumping rate in response to vulva contact. These results suggest that the NSM neurons can also repress pharyngeal pumping rate in response to sensory signals from the genitalia. It is possible that these neurons differentially regulate pharyngeal and genital muscle activity to ensure that feeding and defecation are repressed during mating, and that mating is repressed when the animal is searching for food.

Since the NSMs are required for pharyngeal-mediated suppression of *unc-103(lf)*-induced spicule protraction, we asked if these neurons are also required for food-deprivation suppression of *unc-103(lf)*. We ablated the NSM neurons in *unc-103(dn)* and *unc-103(0)* males and assayed the effects on both well-fed and food-deprived conditions (Figure 10). Ablation of the NSMs in both *unc-103(lf)* backgrounds slightly increased spontaneous spicule protraction in food-deprived males; however this increase was not significantly different than the mock-ablated, food-deprived controls. We hypothesize that although the NSMs are likely involved in food-deprivation suppression, there must be other redundant, non-NSM mediated mechanisms that can suppress sex-muscle excitability.

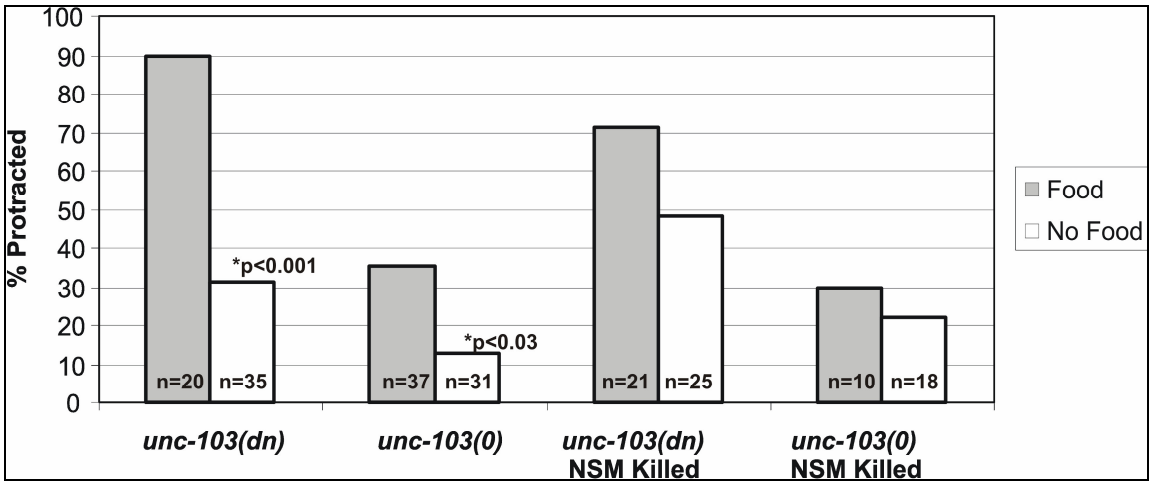

Figure 10. Removal of the NSM neurons is not sufficient to block food-deprivation suppression of *unc-103(lf)*-seizures.
Bar graph displaying the effects of food deprivation on mock-ablated and NSM-ablated males. p-value listed compared to on food control (Fisher's exact test).

Food deprivation suppresses sex-muscle excitability via sensory signals

In addition to NSM-mediated regulation, our data indicates that food deprivation can suppress sex-muscle excitability via non-pharyngeal signals. To dissect these other mechanisms, we reasoned that reduced sex-muscle excitability could be the result of two possibilities: either as a response to decreased ingestion and nourishment of food, and/or as a response to decreased sensation of food in the environment. To differentiate between these two conditions, we asked if exposing males to inedible *E. coli*, i.e. food they can sense but not ingest, is similar to depriving them of food. To generate inedible bacteria, we treated *E. coli* OP50 with the drug aztreonam, which inhibits cell wall septation [91]. Treatment with this drug results in long chains of bacteria (5 to >50µm)

that are too big to fit inside the mouth of *C. elegans* (Figure 11A). To observe effects on wild-type mating efficiency, we separated L4 larval-stage males and placed them on one of three conditions: standard growth plates with food (untreated *E. coli*), with inedible food (*E. coli* treated with azetreonam), and with no food (no bacteria present). Interestingly, we found that wild-type males placed on inedible bacteria for 15hrs prior to the mating efficiency were more likely to successfully mate (40% successful maters) than their food-deprived counterparts (15% successful maters) (Figure 11B).

In addition to measuring mating efficiency, we also measured the effects of inedible bacteria on *unc-103(0)*-induced seizures. The *unc-103(0)* deletion allele caused 38% of males to display spontaneous spicule-muscle seizures on food, whereas only 8% of males placed on the no-food condition displayed the defect. Interestingly, 20% of *unc-103(0)* males placed on plates with inedible bacteria had spontaneous muscle seizures, which is significantly different from both food ($p=0.007$) and no-food ($p=0.017$) conditions (Figure 11C). Taken together, these results suggest that both sensation of food and ingestion of food can influence male mating behavior, possibly through regulating the excitability of the sex muscles.

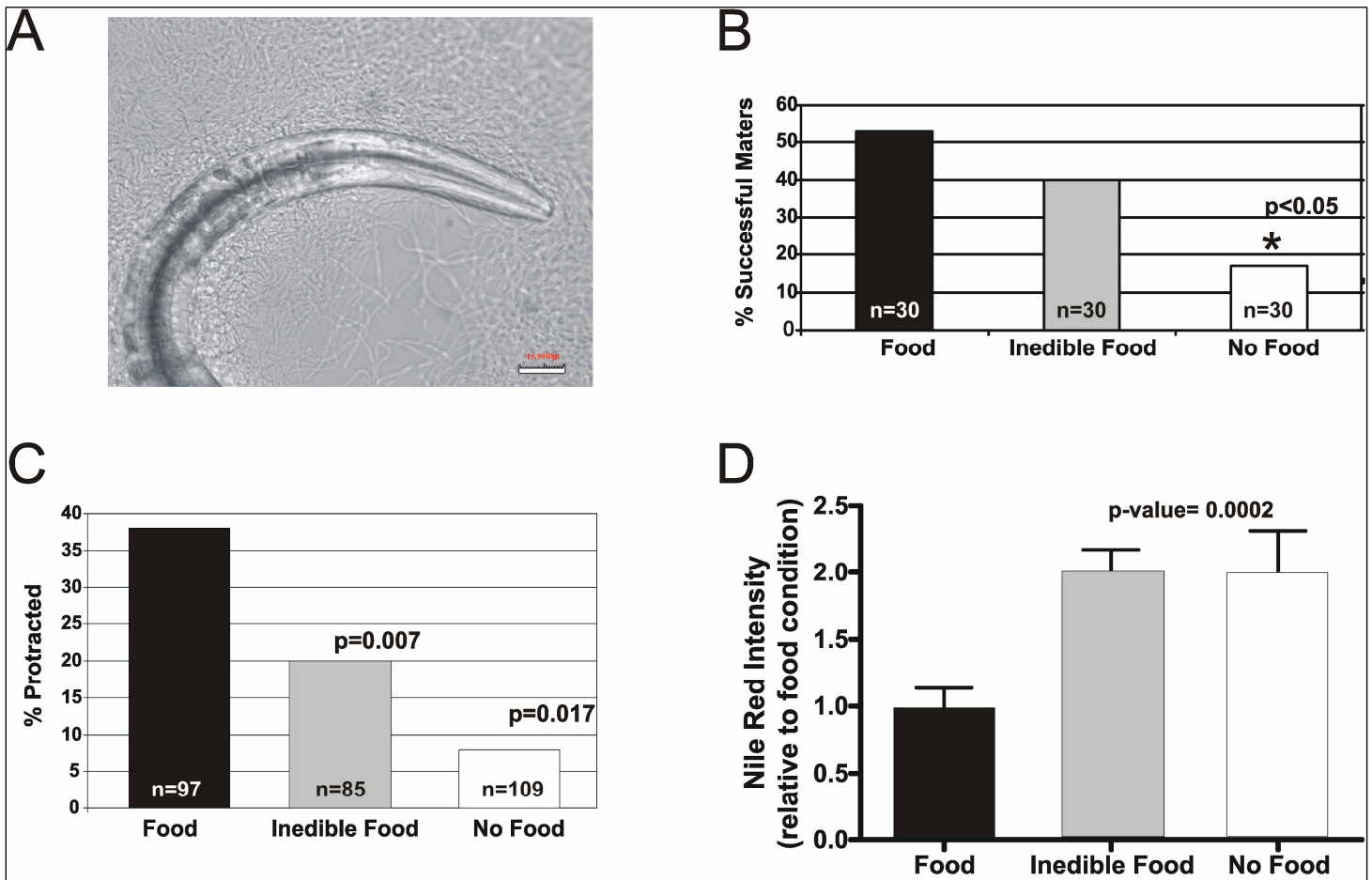

Figure 11. Food deprivation suppresses sex-muscle excitability via both internal and external sensory responses.
(A) Adult male on aztreonam-treated *E.coli* OP50. Scale bar 20µm.
(B) Effects of food, inedible food, and no food on wild-type mating efficiency. p-value Fisher's exact test.
(C) Effects of food, inedible food, and no food on *unc-103(0)*-induced seizures. p-value Fisher's exact test.
(D) Bar graph representing the effects of food, inedible food and no food on Nile Red fluorescent intensity for wild-type males.
(E) Representative Nile Red-stained wild-type males under the three different feeding conditions (scale bar and 23.5µm).
(F) Bar graph representing the effects of different feeding conditions on the mean number of Nile Red-stained fat droplets for wild-type males.
(G) Representative Nile Red-stained *Posm-12:unc-103(gf)* males under the three different feeding conditions (scale bar and 23.5µm).
(H) Bar graph representing the effects of different feeding conditions on the mean number of Nile Red-stained fat droplets. For each condition, 10 males were analyzed for Nile Red staining and the p-values were determined using the Student's t test.

One possible caveat to interpretation of the intermediate phenotype observed with inedible bacteria is that males could still be possibly breaking up and ingesting some bacteria, albeit at a reduced amount. However, males placed on inedible *E. coli* appear to be just as food deprived as males placed on non-treated *E. coli*; both conditions caused the males to have empty intestines and a pale appearance. To determine if males placed on inedible food were similar to food-deprived males more quantitatively, we used Nile Red to visualize internal fat stores [82]. Males were grown on plates containing Nile Red until the L4 larval stage and then placed on one of the three different feeding plates: with *E. coli*, with inedible *E. coli*, and without *E. coli*. We then used two different standards of measurement to compare fat staining between males grown in different conditions: fluorescent intensity and the number of Nile Red-stained fat droplets. Interestingly, we found that both food-deprived males and males placed on inedible bacteria had significant increases in Nile Red intensity staining compared to well-fed males (Figure 11D-E). This result suggests that the fat regulatory mechanisms activated in food-deprived males are similarly activated in males on inedible bacteria. However, in contrast to fat droplet intensity, we found that males grown on aztreonam-treated and non-treated bacteria had significantly higher numbers of Nile Red-stained fat droplets than males grown without bacteria (Figure 11F). Therefore, although males grown on aztreonam-treated bacteria showed similar overall increases in fat-droplet intensity, our data suggests that males grown on aztreonam-treated bacteria are not mobilizing fat stores as quickly as food-deprived males.

To determine why males placed on inedible bacteria appeared as starved as food-deprived males, yet appear to have larger numbers of fat droplets, we hypothesized that sensation of bacteria may block fat mobilization. Specifically, we asked if the reason males on aztreonam-treated bacteria appeared to have more fat was not due to the bacteria being edible, but instead due to the possibility that fat breakdown is inhibited by the sensory perception of food in the environment. To test this, we transgenetically expressed a mutant gain of function UNC-103 K^+ channel in chemosensory neurons using the *osm-12* promoter [92,93], and counted the number of Nile Red fat droplets (Figure 11G-H). The transgenetic UNC-103(gf) K^+ channel contains an A331T change in the sixth transmembrane spanning domain (S6) [63,69,74,94]. The *Posm-12:unc-103(gf)* construct should hyperpolarize chemosensory neurons, reducing their ability to transmit olfactory signals about food in the environment to the rest of the animal. To confirm that the construct reduces the function of chemosensory neurons, we verified that transgenic worms displayed known defects in chemotaxis to the attractant, isoamyl alcohol (Appendix A, Figure A-1) [95,96]. If chemosensation blocks mobilization of fat stores, then *Posm-12:unc-103(gf)* transgenic worms should display decreased levels of Nile Red-stained fat droplets on inedible bacteria. However, if inedible bacteria are indeed edible, then the transgenic worms should show similar levels of fat staining on both inedible and edible conditions. Interestingly, we found that reducing chemosensory function resulted in significantly lower numbers of fat droplets on inedible bacteria (Figure 11G-H). This suggests that aztreonam-treated bacteria are inedible, and that worms placed on these bacteria are not mobilizing fat stores as quickly as males placed

with no bacteria due to chemosensation of the bacteria. Although the increased Nile Red intensity and differences in the number of stained foci raise interesting questions about fat homeostasis that are beyond the scope of this work, our results suggest that males placed on aztreonam-treated bacteria are similarly food-deprived as males cultured with no bacteria.

In addition to visualizing fat storage in males placed on inedible bacteria, we used GFP-expressing bacteria to visualize edibility, and also tested the ability of aztreonam-treated bacteria to sustain developmental growth. As expected, we found that untreated GFP-expressing *E. coli* could be visualized in the isthmus of the pharynx, the grinder, and in the intestines (Figure 12A). Although the worms ground up most of the bacteria before passage to the intestines, for 95% (n=20) of males observed, we detected small amounts of intact GFP-expressing bacteria in the intestine (n=20). In contrast, when we treated the GFP-expressing bacteria with aztreonam, we never detected any fluorescent *E. coli* in the intestines (n=20) (Figure 12B). In 55% of the males placed on inedible bacteria, single, long chains of bacteria were seen trapped in the isthmus of the pharynx, suggesting that few of the aztreonam-treated bacteria are small enough to enter the pharynx, but too large to pass through the grinder and intestines.

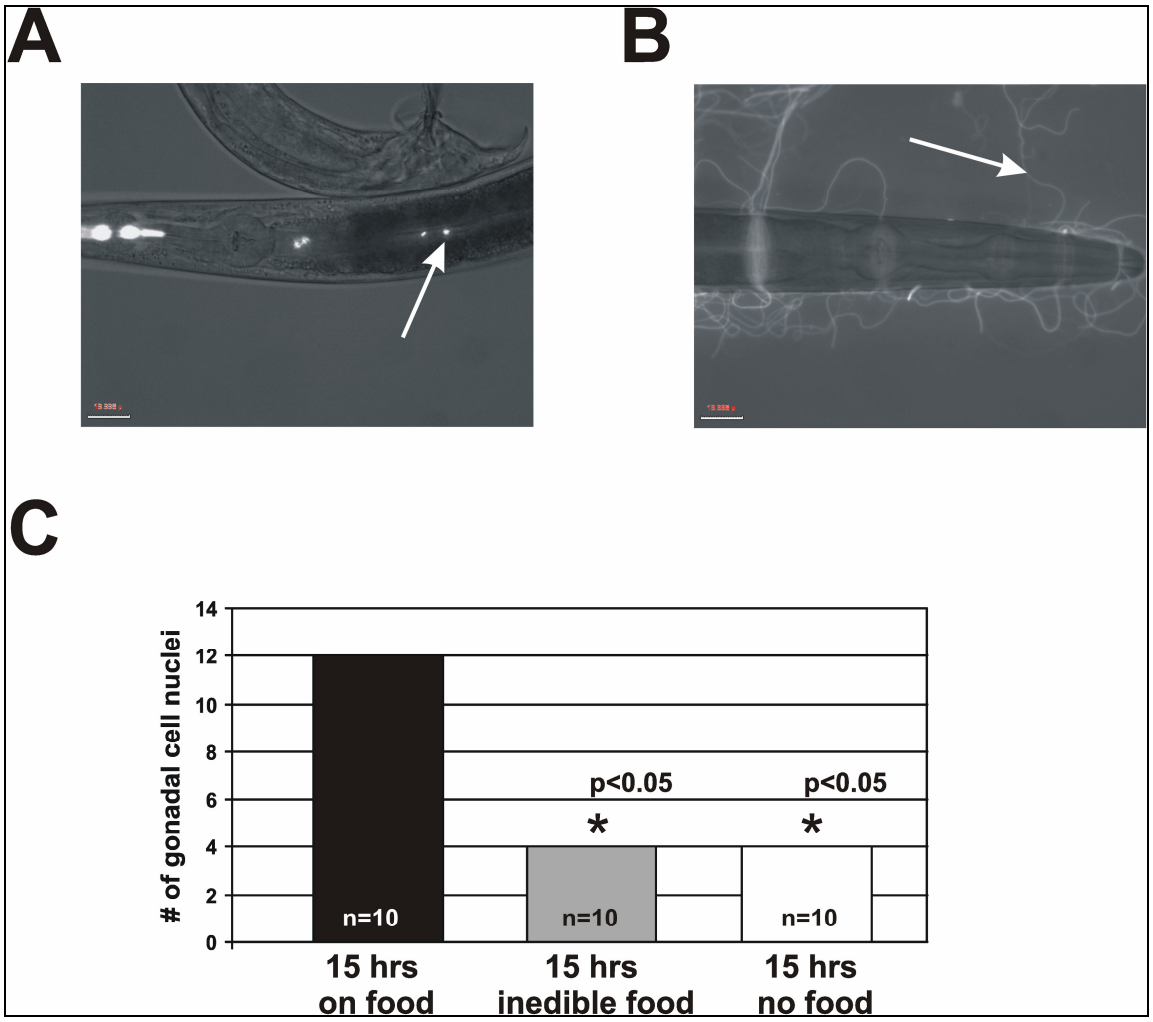

Figure 12: Aztreonam-treated *E. coli* are inedible to *C. elegans*.
(A) Representative image of a male that was fed non-treated GFP-expressing *E. coli*. Arrow points to intact *E. coli* in the intestines (scale bar 20µm).
(B) Representative image of a male that was fed aztreonam-treated GFP-expressing *E. coli*. Arrow points to inedible aztreonam-treated GFP-expressing *E. coli* (scale bar 20µm).
(C) Graph displaying the number of cell nuclei observed in the gonad of L1-stage worms placed on one of the three feeding conditions for 15hrs (p-value Fisher's exact test).

To verify that aztreonam-treated *E. coli* are not digested and cannot sustain growth, we placed starved L1 larva on the three different feeding plates and visualized growth rate during our standard 15hr period by monitoring the number of somatic-gonad cell divisions. *C. elegans* hatch in the developmentally arrested L1 stage and only initiate postembryonic development in the presence of food (bacteria) [97-99]. Thus, we predicted that worms placed on aztreonam-treated *E. coli*, should arrest at L1, similar to worms placed on plates without food. We monitored the number of gonadal cell nuclei to compare the relative developmental stage of worms grown under the three different feeding conditions (Figure 12C). We found that synchronized L1s that were placed on food for 15hrs all contained 12-celled gonads. In contrast, synchronized L1s that were placed on either aztreonam-treated bacteria or no bacteria for 15hrs were arrested, containing 4-celled gonads. Similar to worms grown without bacteria, our results suggest that worms grown on aztreonam-treated bacteria are not ingesting and breaking down nutrients required for developmental growth. Therefore, we conclude that aztreonam-treated bacteria are inedible, and the sensation of the inedible bacteria can block various food-deprivation physiological responses, including mobilization of fat stores and reduced muscle excitability.

Chemosensory neurons suppress sex-muscle excitability

C. elegans uses chemosensation to locate the source of attractive odors (presumably for food), to avoid noxious odors, to determine whether to enter the dauer

diapause stage [100-102]. In addition, males use chemosensation to locate and mate with hermaphrodites [103-105]. To accomplish these tasks, worms use chemosensory neurons that have sensory cilia exposed to the environment, which can react to various chemicals. In regard to male mating, our finding that inedible food can partially block the suppressive effects of food deprivation suggests that chemosensory neurons down-regulate sex-muscle excitability, and their activity is attenuated when food stimulus is present. To determine if lack of sensing *E. coli* partially suppresses sex-muscle excitability, we assayed known chemosensory mutants. We generated double mutants with *unc-103(0)* and *osm-5(p813)* or *osm-9(ky10)*, and assayed the effects on *unc-103(0)*-seizures in the three different feeding conditions (Table 5). *osm-5(p813)* affects the homolog of the murine cystic kidney disease gene, Tg737, that is required for the production of cilia in sensory neurons [105,106], while *osm-9(ky10)* affects a capsaicin receptor homolog involved in sensory responses to a subset of chemical stimuli [67,107]. Neither chemosensory mutant significantly affected the percentage of protracted males under the food or inedible-food conditions. However, we found that both chemosensory mutations significantly increased the percentage of males with seized muscles on the no-food condition. These results suggest that chemosensory neuron function is required to decreased sex-muscle excitability under food-deprived conditions, and their activity is attenuated when a food stimulus is present.

Table 5: Chemosensory mutations block food-deprivation suppression of sex-muscle excitability

Genotype	% Protracted on Food	% Protracted on Inedible Food	% Protracted on No Food
unc-103(0)	39 (102)[a]	20 (85)	9 (101)
osm-5(p813)	0 (10)	0 (10)	0 (10)
osm-9(ky10)	6 (32)	5 (20)	9 (34)
unc-103(0); osm-5(p813)	33 (163)	22 (51)	20[b] (96)
unc-103(0); osm-9(ky10)	41 (59)	28 (40)	21[b] (56)

[a]Numbers in parentheses refer to the number of animals assayed
[b]Significantly different than control No Food condition $p<0.01$, Fisher's Exact Test

To determine which chemosensory neurons suppress sex-muscle excitability in response to food-deprived conditions, we transgenetically expressed the UNC-103(gf) K^+ channel in subsets of neurons using various promoters. Since *osm-12* is expressed in all ciliated neurons, we first used the *osm-12* promoter to determine if we could phenocopy the effects of chemosensory mutations on *unc-103(0)* males. We did not use the promoters for *osm-5* or *osm-9* since their full expression pattern requires coding sequences that may interfere with the UNC-103 K^+ channel fusion. When we expressed *unc-103(gf)* in all ciliated neurons using the *osm-12* promoter, we found that it significantly increased the percentage of protracted males on the no-food condition, similar to the *osm-5* and *osm-9* chemosensory mutations (Table 6).

Table 6: Chemosensory neurons suppress *unc-103(0)*-seizures in the absence of food stimulus

Transgene	Cells Affected	% Protracted on Food	% Protracted on Inedible Food	% Protracted on No Food
None[a]	None	30 (56)[b]	15 (34)	3 (63)
rgEx208 [*Posm-12:unc-103(gf)*]	All ciliated neurons	33 (55)	21 (34)	18[c] (89)
rgEx234 [*Ptax-2:unc-103(gf)*]	AFD, AQR, ASE, ASI, AWC, BAG	36 (89)	24 (41)	21[c] (56)
rgEx205 [*Pocr-2:unc-103(gf)*]	ADF, ADL, ASH,AWA, PHA, PHB	38 (47)	23 (30)	9 (32)
rgEx201 [*Podr-3:unc-103(gf)*]	AWA, AWB, AWC	35 (37)	22 (36)	23[c] (43)

[a]All genotypes contain *unc-103(0)*
[b]Numbers in parentheses refer to the number of animals assayed
[c]Significantly different than the non-transgenic no-food condition p<0.01, Fisher's Exact Test

To narrow down the number of candidate neurons, we then expressed *unc-103(gf)* from more restrictive promoters (Table 6). The promoters and their expression pattern confirmed by YFP reporters were as follows: *Ptax-2* (AFD, AQR, ASE, ASI, ASK, AWC, BAG), *Pocr-2* (ADF, ADL, ASH, AWA, PHA, PHB), and *Podr-3* (AWA, AWB, AWC, PHA, PHB) [108-110]. The *Pocr-2:unc-103(gf)* construct had no significant effect on food-deprivation suppression of *unc-103(0)*-seizures, suggesting that ADF, ADL, ASH, AWA, PHA, and PHB neurons are not required (Table 6). In contrast, both *Podr3:unc-103(gf)* and *Ptax-2:unc-103(gf)* significantly increased the percentage of food-deprived males with seized muscles from 3% to 22% and 24%, respectively. Since the only common neurons that express both of these promoters are the AWC neurons, it is likely that these neurons suppress sex-muscle excitability under food-deprived conditions. We therefore tested if the AWCs are required for food-deprivation suppression of male mating behavior by using the *Podr-3:unc-103(gf)*

construct and through cell ablation (Figure 13A). However, instead of increasing mating potency under food-deprived conditions, we found that removing AWC significantly decreased mating efficiency in well-fed males. During the assays, we noticed that AWC-ablated (genetically or with laser) males rarely encountered the hermaphrodite during the 2hr mating interval (data not shown). This finding is similar to the observations of White and colleagues, which demonstrated that the AWCs are required for male chemotaxis to hermaphrodite pheromones [103]. Therefore, it is likely that AWC functions in multiple aspects of male behavior, and its role in chemotaxis to hermaphrodites could explain why AWC-ablated males have reduced mating efficiency. Additionally, there may be other redundant neurons that can suppress male mating in response to food deprivation in addition to the AWC neurons. To test the role of the AWCs on sex-muscle excitability more directly, we ablated the neurons in *unc-103(0)* males and assayed the number of fed and food-deprived males with seized muscles. Similar to the *Podr-3:unc-103(gf)* and *Ptax-2:unc-103(gf)* experiments, AWC-ablated *unc-103(0)* males had a significantly higher probability of having sex-muscle seizures under food-deprived conditions (24%) than the mock-ablated controls (5%) (Figure 13B). Although we cannot conclude that the AWCs are sufficient or rule out potential roles for other neurons, our data indicates that the AWC neurons regulate the male genitalia in response to food availability.

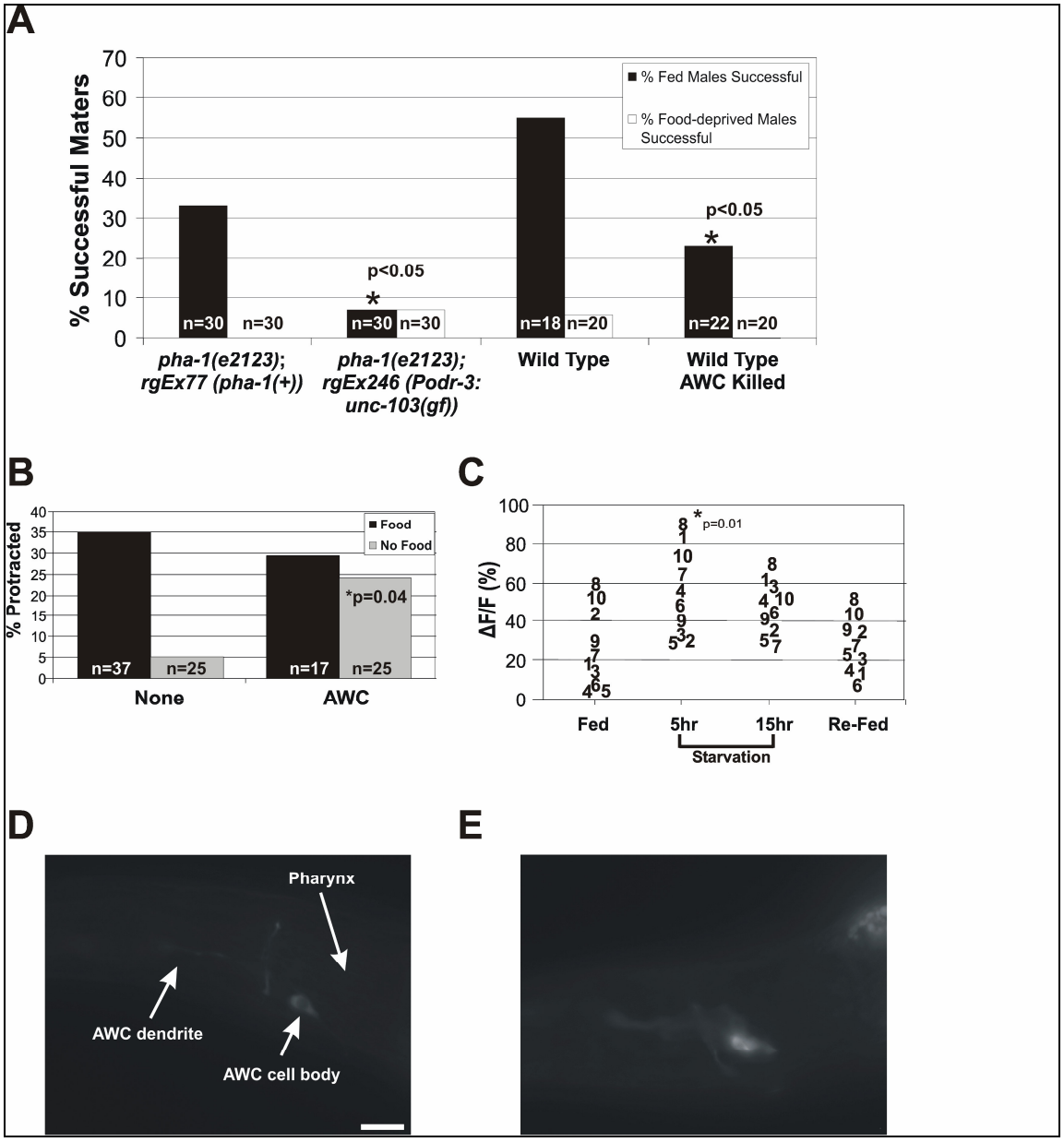

Figure 13. The olfactory AWC neurons regulate sex-muscle excitability in response to environmental conditions.

(A) Graph displaying the effects of *Podr-3:unc-103(gf)* or AWC ablation on male mating efficiency under well-fed and food-deprived conditions. p-value compared to non-ablated control (Fisher's exact test).

(B) Graph showing the effect of laser ablation of the AWC olfactory neurons. The * denotes a significant difference (Fisher's exact test) compared to mock ablated no-food control.

(C) G-CaMP intensity changes in the left AWC neurons of 10 individual males taken after fed, 5hr and 15hr of food deprivation, and re-fed. Each number (1-10) identifies the same male for each interval. The p-value listed signifies a significant difference between Fed and 5-hr food-deprived males.

(D) A representative G-CaMP intensity image of a well-fed male.

(E) A representative G-CaMP intensity image of a 15hr food-deprived male (scale bar 9μm).

The finding that the AWC olfactory neurons are required to suppress sex-muscle excitability in response to food deprivation suggests that these neurons are active when there is no food stimulus, and their activity is attenuated when food is present. Interestingly, Chalasani et al [111] reported that the AWC neurons are activated upon odorant removal. Normally, increases in neuronal and muscular activity are associated with an increased influx of calcium, and G-CaMP is a fluorescent molecule engineered to detect such calcium changes (see Chapter III). Chalasani et al [111] used G-CaMP to demonstrate that AWC activity is repressed in the presence of odorants such as isoamyl alcohol and benzaldehyde, and up-regulated when these odorants are removed. To verify that addition and removal of odorants used by Chalasani and co-wokers corresponded to the presence and absence of *E. coli* in our assays, we used G-CaMP to measure AWC activity in fed, food-deprived, and re-fed conditions (Figure 13C-E). We used the *odr-3* promoter to transgenetically express G-CaMP in the AWC neurons, and used 10, well-fed, adult males to measure fluorescent intensity changes after 5 hours of food deprivation, 15 hours of food deprivation, and after 5 hours of re-feeding. Consistent with the previous findings, we found AWC Ca^{2+} activity increased after removing the male from *E. coli*, and decreased after the animal was re-fed (Figure 13C). These results indicate that the olfactory AWC neurons are activated upon removal of food stimulus from the environment, and result in downstream signaling that reduces sex-muscle excitability. Similar to the NSMs, the mechanism for how AWC activity is communicated to the genitalia is not clear, though it may be through the secretion of neuroactive peptides.

Chapter summary

In Chapter IV, I found that mutation of the muscle protein, tropomyosin (LEV-11) can suppress *unc-103(lf)*-mutant induced seizures by affecting the pharyngeal muscle. The pharynx is a muscular organ the worm uses to ingest food, suggesting that changes in pharyngeal muscle physiology can be communicated to tissues regulating sexual behavior. Consistent with this, mutation of pharyngeal tropomyosin also affects male contact response to hermaphrodites, and the ability to recognize the hermaphrodite vulva. Additionally, I identified the pharyngeal NSM neurons as the likely candidates that can sense pharyngeal perturbations and signal to the sex muscles. These results suggest that NSM neurons use pharyngeal physiological status as a proxy for food availability, and regulate the sex muscles accordingly.

In addition to pharyngeal/NSM regulation of sex-muscle excitability, there must be other redundant mechansisms of suppression since removal of the NSMs is not sufficient to completely block the effects of food deprivation. Interestingly, I found that inedible bacteria, i.e. food they can sense but not eat, partially blocked the suppressing effects of food deprivation on the sex muscles. These results suggest that sensory neuron activity suppresses sex-muscle output when no food stimulus is present. Consistently, I found that the AWC olfactory neurons are required for food-deprivation suppression of sex-muscle seizures. To understand how the AWCs regulate the sex muscles, I demonstrated that AWC activity is up-regulated in food-deprived conditions using the fluorescent Ca^{2+} indicator, G-CaMP. The AWC and NSM neurons, located in the head,

may secrete neuropeptides that can act at a distance as neuromodulators of the sex muscles.

CHAPTER V

FOOD DEPRIVATION SUPPRESSES SEX-MUSCLE EXCITABILITY VIA AN INSULIN-LIKE SIGNALING PATHWAY*

Food deprivation requires the insulin-like receptor, DAF-2

Chemosensory regulation of diverse physiological responses to food deprivation, including fat homeostasis and suppression of seizures suggest that chemosensory neurons may coordinate responses using a common signaling mechanism. We previously reported that the chemosensory AWC and the pharyngeal NSM neurons can suppress mutant-induced sex-muscle seizures, possibly by regulating the secretion of metabolic hormones or neuropeptides. One metabolic signaling pathway known to regulate physiological responses to food is via the release of insulin. In *C. elegans*, insulin-like signaling occurs through the insulin-like receptor, DAF-2, and has been shown to regulate dauer formation, aging, fat homeostasis, and male mate search behavior [104,112-116]. Historically, DAF-2 has been well characterized for its role in regulating physiological responses to food availability, including dauer formation, life-span, and fat homeostasis [112-116]. More recently, there is growing evidence that insulin-like signaling also appears to regulate behavioral responses to food, such as male mate

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*Portions of this chapter are reprinted from LeBoeuf B, Gruninger TR, Garcia LR (2007) Food deprivation attenuates seizures through CaMKII and EAG K^+ channels. PLoS Genet 3: 1622-1632 and from Gruninger TR, Gualberto, DG, Garcia LR (2008) Sensory perception of food and insulin-like signals influence seizure susceptibility. PLoS Genet: in press.

searching behavior [104], thermotaxis to temperatures previously associated with food [38], and associating salt with food-deprived conditions [39]. Therefore, we hypothesized that one mechanism for AWC and NSM regulation of sex-muscle excitability could be through regulating the secretion of insulin-like peptides.

To determine if insulin-like/DAF-2 signaling is required for food-deprivation suppression of sex-muscle excitability, we generated double mutants with *unc-103(0)* and two separate temperature sensitive mutations, *daf-2(e1368ts)* and *daf-2(m41)*. Similar to the chemosensory mutants, we found that mutation of *daf-2* resulted in a significant increase of food-deprived males with spontaneous seizures from 5% to 30% for *daf-2(e1368)* and from 5% to 23% for *daf-2(m41)*, while having no effect on the inedible food condition (Figure 14A). These results suggest that the insulin-like receptor, DAF-2, is required to suppress sex-muscle excitability under food-deprived conditions. Additionally, neither *daf-2* allele significantly affected the percentage of protracted males on inedible-food. Inedible food likely suppresses excitability via a non-chemosensory component, and our data suggests that this component is intact in *daf-2(ts)* mutants since mutant males are still partially suppressed by inedible food.

To test if the NSM's use DAF-2 signaling to suppress *unc-103*-induced seizures, we constructed the triple mutants, *lev-11(rg1); daf-2(e1368) unc-103(0)* and *lev-11(rg1); daf-2(e1368) unc-103(dn)*. Interestingly, we found that the *daf-2(e1368ts)* mutation significantly affected the ability of *lev-11(rg1)* to suppress *unc-103(lf)* spicule protraction (Figure 14B). This suggests that NSM activity likely leads to the release of insulin-like peptides to control genital excitability.

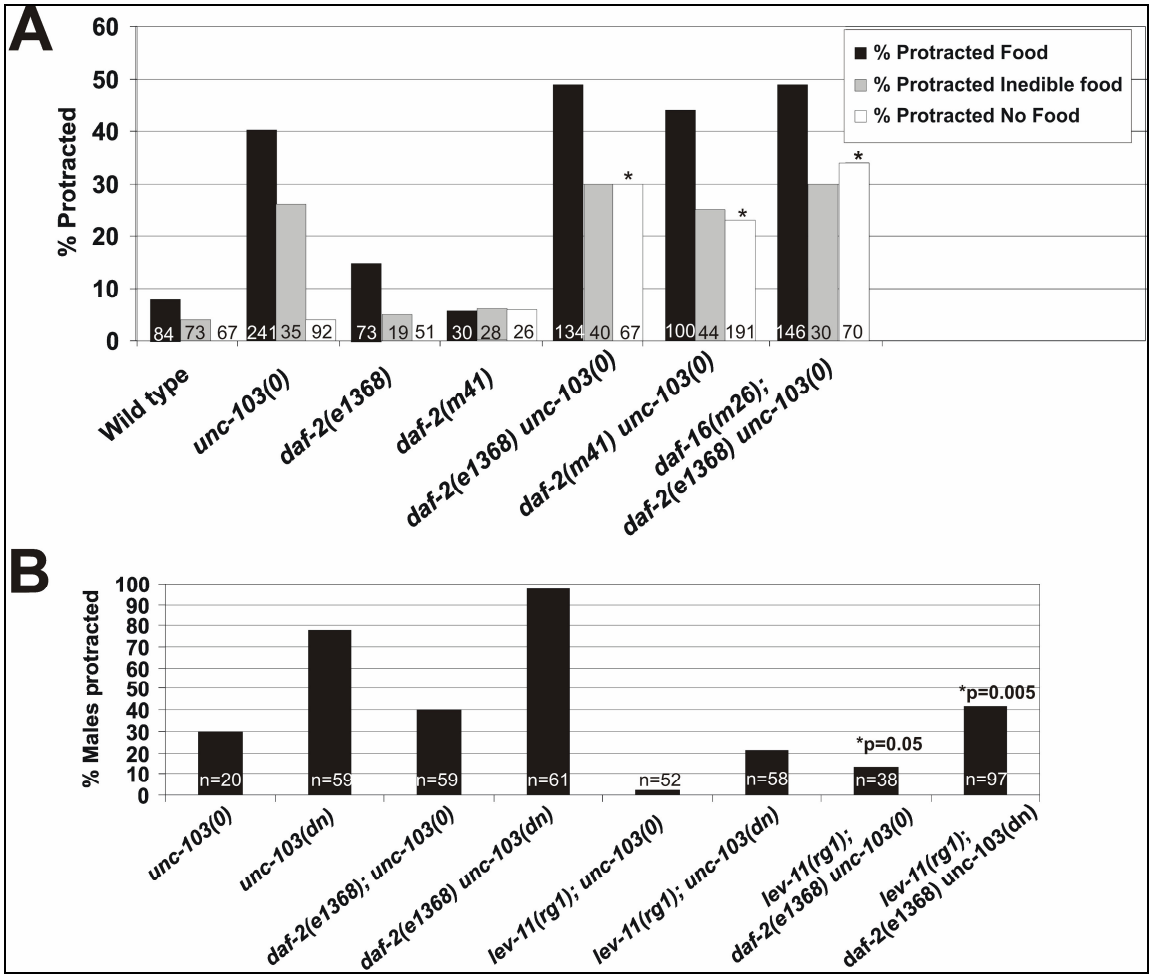

Figure 14. The insulin-like receptor/DAF-2 suppresses sex-muscle excitability under food-deprived conditions.

(A) Graph displaying the effects of *daf-2(lf)* mutations on *unc-103(0)*-seizure susceptibility under food, inedible food, and no food conditions. The * indicates that the p-value is significantly different ($p<0.05$) compared to the *unc-103(0)* control condition (Fisher's exact test).

(B) Graph showing the effect of *daf-2(e1368ts)* on *lev-11(rg1)*-suppression of *unc-103(lf)* seizures.

To test if the AWCs signal through DAF-2, we performed the AWC ablation in *daf-2(e1368) unc-103(0)* double mutants. If the AWC's act through insulin-like signaling to suppress sex-muscle excitability, then we predicted that the AWC ablation should not affect the percentage of food or food-deprived *daf-2(e1368) unc-103(0)* males with seized muscles. However, if they function via separate mechanisms, we predicted that ablation of AWC in the double mutant background should significantly increase the percentage of food-deprived males with protracted spicules. Interestingly, we found that ablation of the AWC neurons in *daf-2(e1368) unc-103(0)* males did not significantly affect the percentage of males with seized muscles on either condition assayed (Figure 15A). Additionally, to mimic the inedible food condition which blocks AWC-mediated suppression of sex-muscle excitability, we soaked Sephadex beads (20-80µM) in odorants previously shown to reduce AWC activity, isoamyl alcohol, benzaldehyde, and 2, 3-pentanedione [111]. We found that beads soaked with AWC-inhibiting odorants increased the percentage of food-deprived *unc-103(0)* males with protracted spicules from 3% to 17%, but did not increase the percentage of food-deprived *daf-2(e1368) unc-103(0)* males with protracted spicules (Figure 15B). Based on these results, we hypothesize that the activity of the olfactory AWC and pharyngeal NSM neurons lead to the secretion of insulin-like signals under food-deprived conditions.

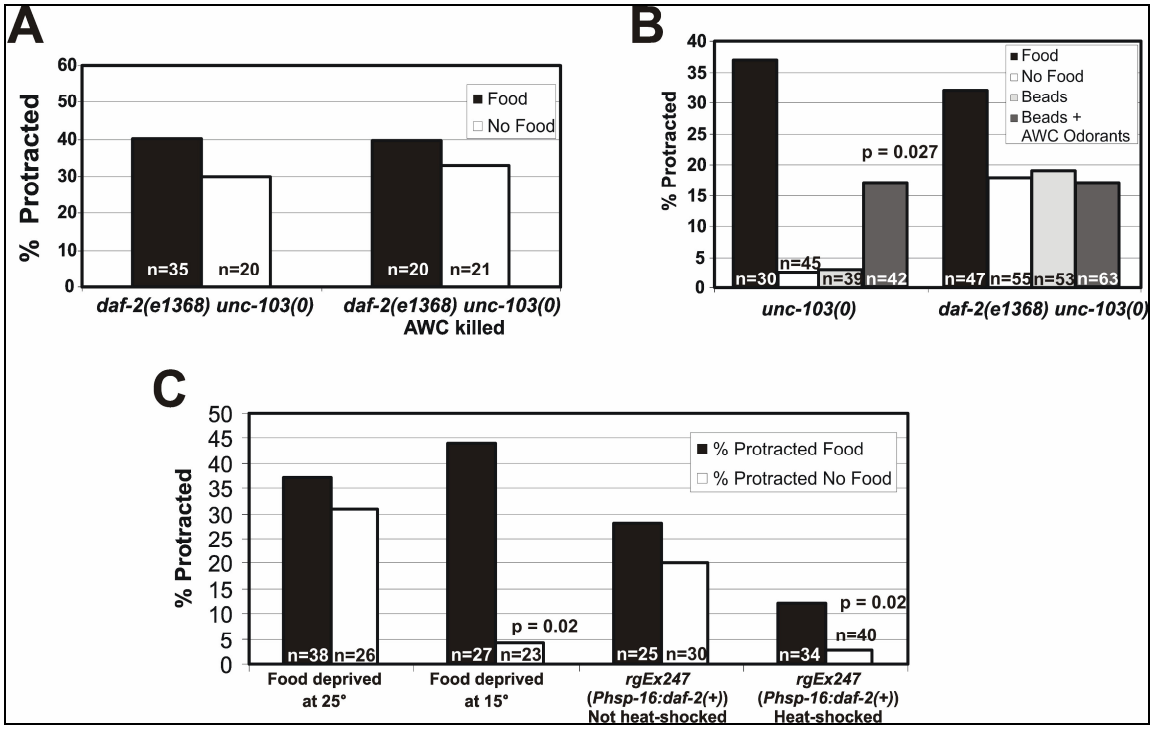

Figure 15: The insulin-like receptor, DAF-2, likely acts downstream of AWC during times of food deprivation.
(A) Graph displaying the effect of AWC ablation on *unc-103(0)*-induced seizures in *daf-2(e1368) unc-103(0)*.
(B) Graph displaying the effect of AWC odorants (10^{-4} isoamyl alcohol, 10^{-7} 2,3-pentanedione, and 10^{-4} benzaldehyde) on *unc-103(0)*-induced seizures in *unc-103(0)* and *daf-2(e1368) unc-103(0)* males. Sephadex G-50 beads were used as a vehicle for the odorants (p-value Fisher's exact test).
(C) Temperature shift assay and heat-shock rescue for *daf-2(e1368ts) unc-103(0)*. Animals were grown at the restrictive temperature (25°C) and then food-deprived at either the restrictive or permissive temperature (15°C). For heat-shock rescue, L4 males were heat-shocked for 35 minutes prior to the assay. The p-values listed were calculated using Fisher's exact test.

DAF-2 regulation of the sex muscles could be occurring either developmentally, to ensure the circuits are set up to respond to food deprivation, or could act acutely at the time of food deprivation. The *daf-2(e1368ts)* mutation has been proposed to be a temperature sensitive mutation that results in a constitutive dauer phenotype at 25°C, whereas at 15°C the dauer phenotype is rescued, presumably due to restoration of wild-

type function of the protein at this temperature [61]. Therefore, to determine if DAF-2 is required developmentally or acutely to regulated sex-muscle behaviors, we raised *daf-2(e1368ts)* males at the restrictive temperature (25°C) and then food deprived them at either the permissive (15°C) or restrictive (25°C) temperatures (Figure 15C). If DAF-2 is required developmentally, then *daf-2(e1368ts) unc-103(0)* males raised at 25°C should display an increase of food-deprived males with protracted spicules regardless of the temperature at which they are food deprived. In contrast, if the receptor is required acutely, then *daf-2(e1368ts) unc-103(0)* mutants should be suppressed for spontaneous seizures when they are food deprived at 15°C. Interestingly, we found that males grown at 25°C and then food deprived at 15°C were suppressed for spontaneous spicule protraction from 44% to 4%, suggesting that DAF-2 acts acutely, at the time of food deprivation to suppress muscle seizures. However, it is possible that *daf-2(e1368)* is not truly wild-type at 15°C, and the effects observed may be due to temperature changes rather than true wild-type function of *daf-2* in this background. To rule out this possibility, we rescued *daf-2(e1368)* in *daf-2(e1368) unc-103(0)* mutants using the inducible heat-shock promoter, *Phsp-16* (Figure 15C). Similar to the temperature shift assay, we found that heat-shocking males 30min prior to our 15hr food deprivation assay resulted in food-deprivation suppression of the sex-muscle seizure phenotype from 12% in well-fed males to 3% in food-deprived males. Additionally, although not quite statistically significant, the *Phsp-16:daf-2(+)* construct appeared to reduce spontaneous spicule protraction in well-fed males as well, suggesting that over-expression of *daf-2* may be able to compensate for loss of *unc-103* function.

Many of the *daf-2(lf)*-mediated behavioral and physiological defects in *C. elegans* are dependent on the FOXO/DAF-16 transcription factor [117-119]. Specifically, to negatively regulate longevity, dauer formation, and stress resistance, previous studies have demonstrated that DAF-2 leads to the phosphorylation of FOXO/DAF-16, which inhibits translocation to the nucleus and transcription of downstream genes [62,120-122]. However, in the absence of DAF-2 signaling, either due to decreased insulin-like ligand stimulation, or due to mutation, DAF-16/FOXO can enter the nucleus and transcribe gene involved in increasing lifespan and stress resistance. Consequently, long-lived and increased stress resistance phenotypes of *daf-2(lf)* animals can be suppressed by mutating DAF-16 so that it can no longer translocate to the nucleus [122,123]. To determine if DAF-2 also acts to inhibit FOXO/DAF-16 mediated signaling to suppresses sex-muscle excitability, we constructed a *daf-16(m26)*; *daf-2(e1368ts) unc-103(0)* triple mutant and assayed the effects of the mutations on the three different feeding conditions (Figure 14A). Interestingly, we found that the *daf-16(m26)* allele did not significantly affect the percentage of males with seized muscles for any feeding condition. This suggests that DAF-2 regulation of sex-muscle excitability, in contrast to its role in dauer formation and longevity, must signal through a DAF-16-independent mechanism. These results are also consistent with DAF-2's role in acute function on the sex-muscles rather than a developmental function.

DAF-2 acts in the sex muscles to suppress spontaneous seizures

In *C. elegans*, the insulin-like receptor/DAF-2 is broadly expressed and regulates multiple physiological functions, including aging, fat regulation, and development [62,112-116,124]. DAF-2 regulates lifespan by acting in the neurons, while DAF-2 activity in the muscles regulates intestinal fat levels [125]. To determine where DAF-2 acts to suppress sex-muscle excitability in response to food deprivation, we generated tissue-specific constructs with wild-type *daf-2* cDNA and injected them into the *daf-2(e1360) unc-103(0)* background (Table 7). We expressed wild-type *daf-2* cDNA in the body-wall/sex-muscles, the pharyngeal muscles, all neurons, and the intestine using the *lev-11*, *tnt-4*, *aex-3*, and *gtl-1* promoters, respectively. We found that only the *lev-11* promoter restored food-deprivation suppression of spontaneous seizures, suggesting that DAF-2 acts in the muscles.

To determine if DAF-2 acts in the sex muscles or body-wall muscles to suppress sex-muscle excitability, we used the *unc-1031E* promoter. Promoter *Punc-103E* is expressed in the sex muscles, 7 pairs of head neurons, and two pairs of pharyngeal neurons [74] (Appendix A, Table A-1). To verify that any effects seen with this construct was due to sex-muscle expression, and not due to neuronal expression we also expressed *daf-2(+)* using the *unc-103F* promoter, which does not express in the sex muscles, but does express in the same neurons expressing *Punc-103E*. Interestingly, we found that the *Punc-103E:daf-2(+)* construct significantly restored suppression of *unc-103(0)*-seizures in food-deprived males. However, the *Punc-103F:daf-2(+)* construct

had no effect, suggesting that DAF-2 is acting in the sex muscles to suppress sex-muscle excitability (Table 7).

Table 7. Tissue-specific rescues of *daf-2(e1368)* in *daf-2(e1368) unc-103(0)* males

Rescue construct[a]	Tissue Expression	% Protracted on Food	% Protracted on No Food
None	None	40 (212)	28 (138)
rgEx178 [*Plev-11:daf-2(+)*]	Body-wall muscles, sex muscles	41 (37)	9[b] (33)
rgEx180 [*Paex-3:daf-2(+)*]	All neurons	14[c] (21)	11 (27)
rgEx179 [*Punc-1031E:daf-2(+)*]	Sex muscles, anal depressor, AIB, RIM, AIY,AVJ, ALA,AVH,NSM, I5	38 (26)	9[b] (22)
rgEx199 [*Punc-1031F:daf-2(+)*]	ALA, ADL, ASK, AVH, AVJ, AIN, AVA, ASJ, SMDD, SIA, ADE, AVD, I2, NSM, IL, IL2, OLL, URA, ASH, AVD, AUA,AUA, SIA, OLQ, RIV, URY, AIN, AIA, SPC, PCA, PCB, HSN, ray 1,2,3,4,6,9	46 (48)	29 (38)
rgEx236 [*Pgtl-1:daf-2(+)*]	intestines	48 (33)	34 (41)
rgEx237 [*Ptnt-4:daf-2(+)*]	pharyngeal muscles	41 (49)	38 (21)

[a]Background strain is *daf-2(e1368) unc-103(0)*
[b]Significantly different than the non-transgenic no-food control, Fisher's exact test
[c]Significantly different than the non-transgenic food control, Fisher's exact test

In addition to acting in the sex muscles, we identified a potential role for neuronal DAF-2 signaling in sex-muscle regulation. Surprisingly, we found that panneuronal rescue of *daf-2* significantly suppressed spontaneous *unc-103(0)* muscle seizures in well-fed males (Table 7). This suggests that increased DAF-2 signaling in the neurons can activate mechanisms that can compensate for loss of UNC-103 function. However, this regulation of sex-muscle excitability is distinct from DAF-2 regulation during food-deprived conditions, since no further decrease is seen in food-deprived males. Although neuronal DAF-2 signaling may influence the sex muscles on food, our

results indicate that it is sex-muscle specific insulin-like signaling that reduces seizure susceptibility during food-deprived conditions.

CaMKII and EAG K^+ channels act in the sex muscles to suppress cell excitability

We previously implicated a role calcium/calmodulin-dependent kinase II (CaMKII)/UNC-43 in regulation of sex-muscle excitability in response to food deprivation (Chapter III). The *unc-43(sy574)* allele of CaMKII causes spontaneous sex-muscle seizures that cannot be alleviated by food deprivation, suggesting that CaMKII may act with insulin-like receptor/DAF-2 to compensate for *unc-103(0)*-mediated increased excitability (Table 7). To determine if mutations in *unc-43* also block food-deprivation suppression of *unc-103(0)*-induced seizures, we constructed double mutants with *unc-103(0)* and two different alleles of *unc-43*, *unc-43(sy574)* and *unc-43(e408).* Interestingly, we found that food depriving *unc-103(0)*; *unc-43(sy574)* males reduced spontaneous spicule protraction from 88% to 62% (a percentage similarly displayed by *unc-43(sy574)* single mutants), whereas *unc-103(0)*; *unc-43(e408)* males showed no significant suppression after food deprivation (Table 8). The *unc-43(sy574)* allele induces spontaneous muscle seizures due to a point mutation that changes a glycine to a glutamate at amino acid 170, near the substrate recognition domain. The *unc-43(e408)* allele is a point mutation that results in a serine to leucine change at amino acid 179, also near the substrate recognition domain [66]. Consequently, these alleles may affect interaction with downstream effectors differently. *unc-43(e408)* induces severe

pleiotropic behavioral abnormalities not observed in *sy574* animals, suggesting that the *unc-43(e408)* kinase activity is more severely affected than the *unc-43(sy574)* kinase. We hypothesize that the *sy574*-kinase may still retain enough function to suppress the *unc-103(0)* phenotype in response to food-deprivation, however is unable to suppress its own induced spontaneous protraction defect. In contrast, the *e408* allele completely disrupts UNC-43's ability to transmit food-deprivation signals.

To determine if CaMKII/UNC-43, similar to insulin-like receptor/DAF-2, acts in the sex muscles to suppress sex-muscle excitability in response to food deprivation, we expressed wild-type *unc-43* in the spicule muscles from the *Punc-103E* promoter in *unc-103(0)*; *unc-43(e408)* males. Interestingly, we found that expressing wild-type *unc-43* from this promoter restored food-deprivation suppression of sex-muscle seizures from 50% to 13% (Table 8). These results suggest that CaMKII/UNC-43 may act downstream of DAF-2 signaling to suppress sex-muscle excitability.

Previous biochemical, pharmacological, and genetic studies in *Drosophila* and *C. elegans* suggest that CaMKII/UNC-43 can bind and activate EAG-like K^+ channels/EGL-2 [66,126-128]. Additionally, EAG-like K^+ channels are encoded by another sub-family of genes related to ERG-like K^+ channels/UNC-103 and these channels are co-expressed with *unc-103* in the male sex muscles [127,129]. Therefore, we tested whether CaMKII could lead to decreased sex-muscle excitability by activating the worm EAG-like K^+ channel, EGL-2 [127]. On food, *unc-103(0); egl-2(0)* males behaved similarly to *unc-103(0)* single mutants (Table 8). However, we found that the *egl-2* deletion allele significantly increased the percentage of food-deprived males with

sex-muscle seizures. To determine if EGL-2 also acted in the sex muscles to suppress excitability under food-deprived conditions, we expressed wild-type *egl-2* cDNA from the body-wall/sex muscle *lev-11* promoter and the sex-muscle *unc-103E* promoter. We found that both the *lev-11* muscle and *unc-103E* sex-muscle rescue-constructs restored food-deprivation suppression of *unc-103(0)* from 36% of food deprived males displaying seizures to 9% and 4%, respectively. These results identify CaMKII and EAG K^+ channels as components of a signaling pathway that reduces muscle excitability under food-deprived conditions.

Table 8. CaMKII/UNC-43 and EAG K+ channels/EGL-2 suppress *unc-103(0)*-seizures

	% spicule protracted males (n)	
Genotype[a]	**NGM plates + *E. coli* (OP50)**	**NGM plates + no bacteria**
Wild Type	5 (38)	0 (26)
unc-103(0)	36 (44)	9 (23)[b]
unc-43(sy574)	44 (66)	50 (44)
unc-43(e408)	0 (30)	n.d.
unc-103(0); *unc-43(sy574)*	88 (74)	62 (71)[b]
unc-103(0); *unc-43(e408)*	61 (31)	50 (22)
unc-103(0); *egl-2(0)*	39 (85)	36 (67)
unc-103(0); *egl-2(0)* *rgEx175* [$P_{lev\text{-}11}$*::egl-2(+)*]	35 (34)	9 (22)[b]
unc-103(0); *egl-2(0)* *rgEx173* [$P_{unc\text{-}103E}$*::egl-2(+)*]	39 (28)	4 (23)[b]
unc-103(0); *unc-43(e408)* *rgEx172* [$P_{unc\text{-}103E}$*::unc-43(+)*]	38 (45)	13 (40)[b]

[a]Strains contain *him-5(e1490)*.
[b]p-value<0.05 compared with NGM plates + *E. coli* of the same genotype; Fisher's exact test

PLC-γ may act downstream of DAF-2

Since DAF-2 regulation of sex-muscle excitability does not signal through the canonical FOXO/DAF-16 signaling pathway, we looked for other downstream targets that could link DAF-2 signaling with CaMKII and EAG K^+ channels. Previous work by others has demonstrated that insulin-like growth factor I (IGF-1) can activate CaMKII signaling pathways in human neuroblastoma and rat hippocampal neurons, and this occurs via increased cystolic Ca^{2+} from PLC-γ/IP_3 activity [130-132]. Additionally, DAF-2 contains a SH2-binding/PLC-γ motif [116]. To test if PLC-γ acts downstream of insulin-like signaling in *C. elegans* to suppress sex-muscle excitability, we used RNAi to reduce the expression of the worm homolog of PLC-γ, PLC-3 (Figure 16A). RNAi of PLC-3 significantly increased the percentage of food-deprived males with spontaneous seizures from 10% to 27%, whereas RNAi of another PLC-homologue, PLC-2 had no significant effect. To test the role of PLC-3 more rigorously, we constructed an *unc-103(0)*; *plc-3(sy698)* double mutant (Figure 16A). *plc-3(sy698)* is a Tc1 insertion that likely behaves like a genetic null [58]. Similar to the RNAi data, we found that mutation of *plc-3* caused a significant increase of food-deprived males with protracted spicules from 5% to 23% (Figure 16). Therefore, it is possible that insulin-like/DAF-2 signaling may lead to activation of CaMKII and EAG K^+ channels via PLC-γ, and result in decreased sex-muscle excitability.

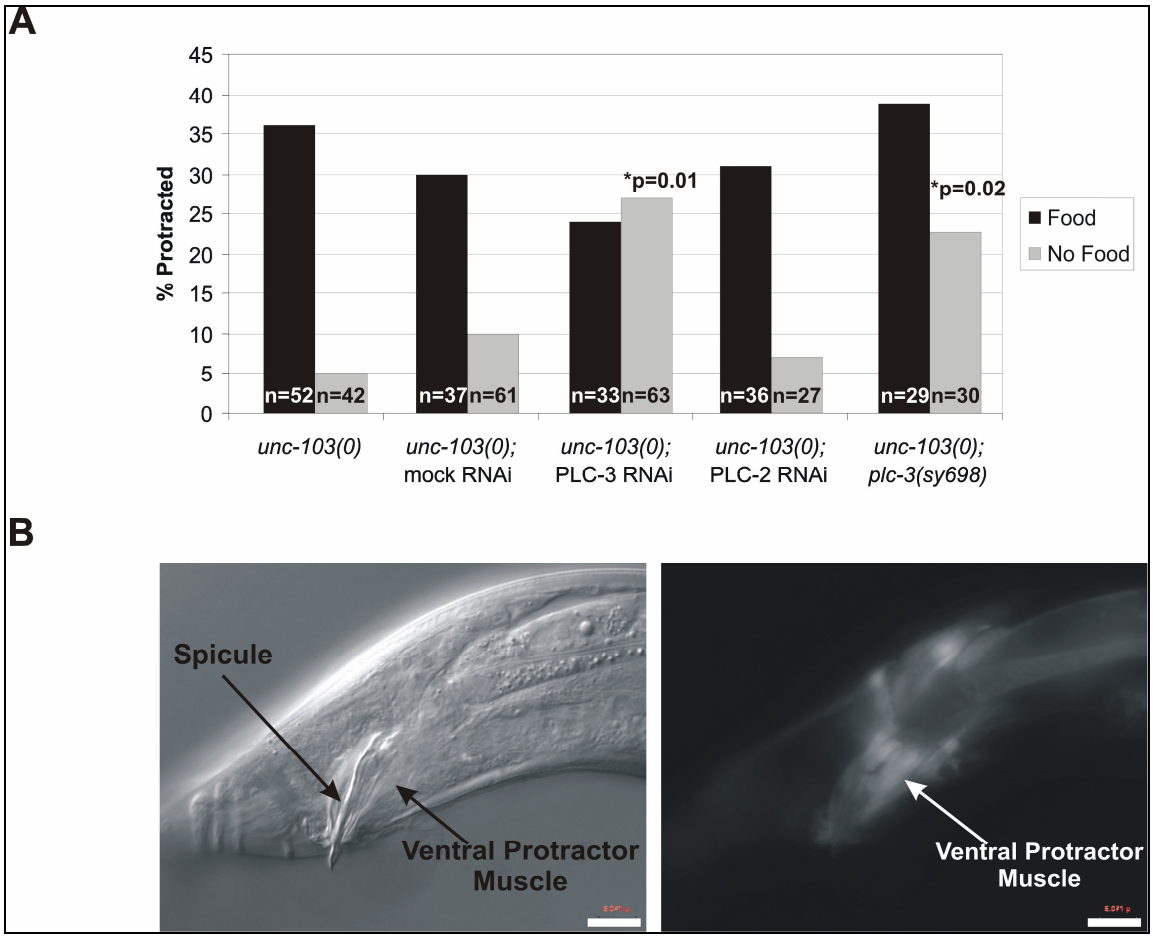

Figure 16. Phospholipase C-γ/PLC-3 is required for suppression of *unc-103(0)*-seizures.
(A) Graph displaying the effects of PLC-3, PLC-2 RNAi, and *plc-3(sy698)* on *unc-103(0)*-seizure susceptibility on food and no-food conditions. p-value for PLC-3 RNAi relative to *unc-103(0)* Mock RNAi no-food control, p-value for *unc-103(0); plc-3(sy698)* relative to *unc-103(0)* no-food control (Fisher's exact test).
(B) *Pplc-3*:YFP expression pattern in young adult male. The ventral protractors are labeled. Scale bar 6µm.

To determine if PLC-3 could be regulating food-deprivation suppression of sex-muscle excitability by acting in the same cells (sex muscles) as *daf*-2, *unc-43*, and *egl-2*, we determined the expression pattern of *plc-3*. In males, *plc-3* was previously shown to

express in the seminal vesicle valve cell and the vas deferens [133]. Using a larger upstream promoter region, we found that our 4.4kb *plc-3* promoter-YFP construct also expressed in the ALA neuron (data not shown), the male retractor muscles, and the ventral protractor muscles (Figure 16B). Taken together with mammalian cell culture studies, these results suggest that under food-deprived conditions, DAF-2 signaling stimulates an increase in cystolic Ca^{2+} via PLC-3 activation of IP3 and results in activation of CaMKII.

Since EAG-like K^+ channels (EGL-2) are also required in the sex muscles for food-deprivation suppression, and are a substrate for CaMKII [126-128], it is likely that activation of CaMKII could then lead to decreased muscle excitability by activating these channels and hyperpolarizing the cell. To test this directly, we generated a *daf-2(e1368) unc-103(0); egl-2(gf)* triple mutant and assayed the number of food-deprived males with seized muscles. We hypothesized that a constitutively active EGL-2 K^+ channel should suppress effects of *daf-2(lf)* mutants on spicule output if these channels act downstream to decrease cell excitability. We found that this was the case, as *daf-2(e1368) unc-103(0); egl-2(gf)* males were suppressed from 38% (n=45) to 8% (n=45) by food deprivation, which was significantly different than the 28% (n=43) observed for food-deprived *daf-2(e1368) unc-103(0)* males (p=0.03; Fisher's exact test).

Chapter summary

In Chapter V, I identified a novel pathway regulated by the insulin/IGF-1-like receptor, DAF-2, that can suppress muscular output in response to food deprivation. Insulin-like signaling has been implicated in many physiological responses related to food availability, including lifespan and metabolism. In addition to regulating these lasting effects, I demonstrated that insulin-like signaling can also mediate acute changes in muscle excitability. Specifically, the DAF-2/insulin-like receptor is required in the sex muscles to suppress *unc-103(lf)*-induced seizures during food deprived conditions. This finding identifies an active role of insulin-like signaling during caloric restriction that does not involve the canonical FOXO/DAF-16 pathway. Instead, I provide evidence that suggests this response by insulin-like signaling involves activation of the downstream molecules phospholipase C-γ, calcium/calmodulin-dependent kinase II (CaMKII), and *ether-a-go-go* K^+ channels. These molecules likely identify a novel mechanism used by organisms to regulate multiple physiological responses to changing environmental conditions.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Summary of experiments

The purpose of this study was to dissect, at the cellular and molecular level, how environmental changes affect an organism's ability to carry out specific behavioral tasks. I used the regulation of *C. elegans* male sex-muscle excitability under well-fed and food-deprived conditions to determine how feeding state regulates sensory-motor circuits. *C. elegans* males appear to use signals from the feeding organ, the pharynx, and from chemosensory neurons to determine food availability and regulate the sex muscles accordingly.

I found that mutation of the pharyngeal muscle protein, tropomyosin, and RNAi of another pharyngeal muscle protein, troponin T, can suppress mutant-induced sex-muscle seizures. Pharyngeal muscle physiology likely influences the male genitalia by signaling through the pharyngeal neurosecretory motor neurons (NSMs), which have sensory endings attached to the adjacent muscles cells and are thought to act as proprioceptive/stretch receptors [90]. Thus, it is possible that reduced food intake induces changes in pharyngeal physiology, and these changes are sensed by the NSMs. Additionally, I determined that the olfactory AWC neurons are up-regulated under-food

deprived conditions, and are required for food-deprivation suppression of sex-muscle excitability.

It is not clear exactly how the NSMs and AWC neurons communicate to the genitalia; however, it is possible that they act through controlling the release of insulin-like peptides. This supported by our findings that the insulin-like receptor, DAF-2, is required in the sex muscles for suppression of *unc-103*-seizures by *lev-11(rg1)* and by food deprivation. Additionally, I have demonstrated that insulin-like signaling results in decreased excitability not through canonical FOXO/DAF-16 signaling, but via the activation of other downstream molecules, including phospholipase C-γ, CaMKII, and EAG K^+ channels. These findings identify a specific signaling mechanism that may be utilized in higher organisms to regulate sensory motor circuits in response to food deprivation

Sex-muscle specific UNC-103 and pharyngeal specific LEV-11 differentially regulate male sex-muscle excitability

I have found that *C. elegans* male mating behavior is suppressed by food deprivation. One reason for this response may be due to down-regulation of sex-muscle excitability, since food-deprivation also reduces calcium-induced sex-muscle response to ACh agonists. To understand how male sex-muscle output is regulated at the molecular level, a previous screen for mutants with spontaneous sex-muscle seizures identified a loss-of-function allele in *unc-103* [56]. *unc-103* encodes a K^+ channel related to

Drosophila seizure/erg and human ether-a-go-go delayed inward rectifying voltage-gated K^+ channel (HERG) [51,134,135]. In flies, ERG mutations cause seizures, and, in humans, perturbations in HERG result in cardiac arrhythmias [50,136,137]. I have shown that *unc-103* inhibits spontaneous sex-muscle output in *C. elegans* by acting in the sex muscles, suggesting that loss-of-function mutations cause spontaneous protraction by increasing sex-muscle excitability. Interestingly, I found that the spontaneous seizure phenotype of *unc-103(lf)* mutants can be affected by changes in the neuromuscular organ controlling food ingestion, the pharynx.

The spontaneous seizure phenotype caused by *unc-103(0)* and *unc-103(dn)* is suppressed by the *rg1* allele in the *lev-11* tropomyosin gene. This mutation affects all isoforms of tropomyosin, and disrupts multiple behaviors such as pharyngeal pumping, egg laying, locomotion, contact response, male turning, and vulva location. Surprisingly, spicule muscle contractions during mating are normal, suggesting that muscles other than the sex muscles can regulate male-mating behavior. The suppression of *unc-103(lf)*-seizures can be alleviated by wild-type tropomyosin in the pharyngeal muscle and intestine. It is possible that tropomyosin could be acting in either tissue to influence the sex muscles; however, the pharynx is the likely site of action. This is supported by my finding that pharyngeal-specific troponin T (TNT-4) and the pharyngeal NSM neurons are required for *lev-11(rg1)* suppression of *unc-103(lf)*. I propose that pharyngeal regulation is likely not specific to just the sex muscles, but rather a general down-regulation of all mating behaviors. The finding that pharyngeal *lev-11* can also regulate another step in male mating, vulva location behavior, supports this model.

The dominant loss-of-function allele *unc-103(dn)* results in a higher penetrance of males displaying sex-muscle seizures than the *unc-103(0)* deletion allele. Additionally, unlike *unc-103(0)*, *unc-103(dn)* is not completely suppressed by *lev-11(rg1)* and suppression is only partially mediated via the NSM neurons. I hypothesize that the difference between these alleles is due to the *sy557* allele interfering with compensating mechanisms in the sex muscles. This is supported by my observations that rescuing *lev-11(rg1)* in either the body-wall and sex muscles or in the pharyngeal muscles and intestine partially restores the *sy557*-mediated phenotype. In contrast, only pharyngeal rescue of *lev-11(rg1)* can restore *unc-103(0)*-mediated seizures. Therefore, it is likely that *lev-11(rg1)* counteracts defects caused by *unc-103(dn)* in muscles, in addition to activating inhibitory signals from the pharynx, whereas in *unc-103(0)* males, the increased cell-excitability is alleviated by altering pharyngeal *lev-11*.

The pharyngeal NSM neurons regulate male sex-muscle excitability

I have found that *C. elegans* male mating behavior and genital excitability is dependent on the feeding state of the male. Similarly, Lipton et al previously described a link between feeding and male-mate searching behavior [104]. They demonstrated that *C. elegans* males will stay in environments that include food (*E. coli)* and potential mating partners (hermaphrodites). When hermaphrodites are not present, a well-nourished male well leave the food source in an apparent search for a mate, whereas a food-deprived male will not leave the food source. However, how feeding status is

communicated to circuits controlling male reproductive behaviors was unknown. Here I provide data that indicates neurosecretory motor neurons (NSMs) located in the pharynx can signal to the genitalia in response to changes in the muscular feeding organ.

Others have hypothesized that the NSM neurons might sense bacteria in the pharyngeal lumen or sense changes in the pharyngeal muscle via stretch receptors, identifying these neurons as candidates to signal the presence or absence of food to the rest of the animal [90]. Consistent with this idea, Sawin and coworkers [138] found that food-deprived animals lacking the NSM neurons failed to reduce their locomotion upon encountering food. I have provided additional evidence for NSM regulation of behavioral responses to food status by demonstrating that pharyngeal-mediated suppression of *unc-103(lf)*-seizures requires the NSMs. We postulate that the NSM neurons use pharyngeal physiological status as a read-out for food availability and signal to the rest of the animal; specifically, they can suppress mating-specific behaviors when the feeding apparatus is not functioning properly (Figure 17). Interestingly, work done by You and colleagues demonstrated that the pharyngeal muscle undergoes distinct, muscarinic-regulated, physiological changes during starvation [139]. It is therefore possible that the NSMs can directly sense these induced pharyngeal changes.

My data identifies a role for the NSMs in controlling pharyngeal muscle contractions. Although the NSMs are pharyngeal neurons and secrete neurotransmitters (i.e. serotonin) that are known to regulate pharyngeal pumping, previous work determined that the NSMs are not required for normal pharyngeal pumping rate in the presence of bacteria [140]. One possible explanation for this is that these neurons may

regulate pumping rate only in specific biological contexts. The analysis of male mating mutants supports this hypothesis; I found that NSM activity is required to repress pharyngeal pumping rate during mating behavior. Additionally, the decrease in pumping rate seen in *lev-11(rg1)* and *unc-103(lf)* males when the NSMs are ablated suggests these neurons are also functioning to increase pumping rate in mutants. These observations suggest that the NSM neurons up-regulate pharyngeal pumping when pharyngeal physiology changes, and down-regulate pumping during periods of mating. I hypothesize the functional consequence of such regulation may ensure that male does not defecate during periods of mating.

I speculate that in *lev-11(rg1)* males, the tropomyosin mutation causes the pharynx to activate the NSM neurons, which not only results in increased pharyngeal pumping, but also attenuates sex-muscle excitability (Figure 17). *lev-11(rg1)* does not reduce sex-muscle excitability by simply reducing pharyngeal pumping rate or reducing food intake, since *lev-11(rg1)*; *unc-103(lf)* double mutants still pumped at wild-type levels and do not show the characteristic pale appearance of starved animals. Additionally, ablation of the NSM neurons has no effect on spicule insertion behavior in well-fed males, suggesting that these neurons are not required for the development of the spicule muscles and neurons. It is therefore more likely that the NSMs suppress sex-muscle excitability by releasing neurotransmitters or neuropeptides in response to pharyngeal changes.

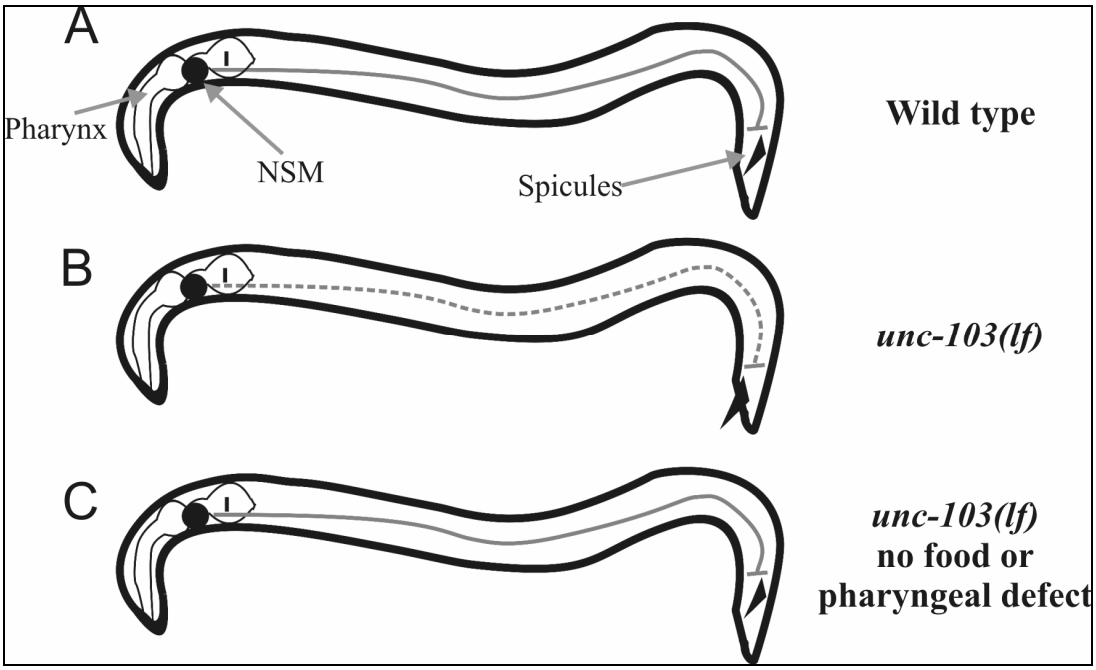

Figure 17. Pharyngeal defects or the absence of food induces the NSMs to downregulate excitable cells in the male genitalia.
(A) In wild type, the NSM will down-regulate the male genitalia during periods when food is not available.
(B) Loss of *unc-103* function in the male genitalia causes the male spicule neurons and muscles to be hyper-excitable.
(C) In *unc-103(lf)* animals, starvation or disrupting the pharyngeal system via mutations can up-regulate the NSMs to attenuate the male genitalia.

The NSM neurons have been reported to use a plethora of neurotransmitters and neuropeptides, including serotonin, the FMRFamide-related protein FLP-4, the neuropeptide-like proteins NLP-13, NLP-18, and NLP-19, and the insulin-like peptide INS-1 [72,90,141-145]. Serotonin is not the likely transmitter used by the NSMs since the interaction between *lev-11(rg1)* and *unc-103(lf)* does not require *tph-1* encoded tryptophan hydroxylase (my unpublished observation). This is consistent with other

work that has suggested that serotonin acts positively on the mating circuit to increase mate searching, response to hermaphrodites, and ventral turning. Similarly, *ins-1* is not required for food-deprivation suppression of *unc-103(lf)*, suggesting this peptide is also not used to suppress sex-muscle excitability (my unpublished observation). At this time, how the NSM communicates with the male genitalia is unknown; regulation could occur through the secretion of other neuropeptides or through regulating downstream neurons that secrete insulin-like peptides.

It should be noted that altering the pharyngeal muscle is distinct from food deprivation, as tropomyosin mutants can still sense and ingest food from the environment. This is supported by the observation that the NSM neurons are not required for food-deprivation suppression of *unc-103(lf)*-induced seizures. I hypothesize that food deprivation suppresses sex-muscle excitability via multiple mechanisms. Specifically, food deprivation likely circumvents the requirement for the NSM by activating other neurons that act on the same molecules downstream of NSM-signaling (i.e. insulin-like molecules). It is plausible that lack of nourishment of food contributes to food-deprivation suppression of muscle excitability, since food-deprivation could induce cellular stress and/or damage. However, in my assays, I found that this is likely not occurring since the periods of food-deprivation did not increase the catabolism of cellular components, or autophagy, as measured by a GFP-tagged LGG-1. Consequently, food deprivation must be acting through other sensory mechanisms in addition to pharyngeal/NSM signaling.

Chemosensory neurons regulate male sex-muscle excitability

To dissect the other pathways that suppress *C. elegans* male mating behavior in response to food deprivation, I demonstrated that food availability can regulate *C. elegans* sex-muscle excitability via two general mechanisms, through chemosensation of food and ingestion of food. It is likely that the non-chemosensory/ingestive component involves the pharyngeal muscle and NSM neurons. In addition to ingestion of food, I demonstrated that food availability can influence sex-muscle excitability through activity of chemosensory neurons. Interestingly, I found that food-deprivation suppression in *unc-103(0)*-seizures can be partially blocked by disrupting chemosensory function, or by placing the males on inedible food.

The ciliated olfactory AWC neurons in the head are required to suppress sex-muscle excitability in response to low-food conditions. Previous work has demonstrated that AWC activity is up-regulated when food and food-odorants are removed from the environment, and repressed when the odorants are present [111]. Consistent with these observations, I found that prior to food deprivation, males displayed decreased AWC activity which markedly increased as the animal was food deprived, and the dropped again when males were re-fed. Thus, similar to the NSMs, AWC activity is initiating signals that down-regulate sex-muscle excitability.

In hermaphrodites, the AWC neurons mediate attraction to at least 5 volatile odors, and also function to increase turning probability during local search behavior. Local search behavior allows animals that have been removed from a food source to

explore limited areas before dispersing and searching for food elsewhere [95,100,146]. The AWCs likely regulate turning probability via glutamate-mediated synapses shared with AIB and AIY interneurons, which can signal to the SMD and RIV head motor-neurons [111,146]. In males, the AWCs have also been shown to mediate attraction; however, in this case, they appear to direct well-fed males toward hermaphrodite pheromones. It is possible that in males, AWC-mediated attraction to hermaphrodites may involve similar mechanisms used by these neurons in hermaphrodites to mediate chemotaxis to chemical attractants. The AWCs likely regulate turning probability through glutamate-mediated synapses to the AIB and AIY interneurons, which can signal to the SMD and RIV head motor-neurons [111,146]. However, in my studies, it is not exactly clear how the AWCs communicate to the male genitalia. Although there are clear sexually dimorphic differences in AWC function, both males and hermaphrodites appear to use these neurons to mediate attraction to an odorant source [95,100,103,146]. Based on my AWC-G-CaMP experiment, I propose that, in males, AWC likely mediates attraction to food odorants as well as hermaphrodite pheromones.

I hypothesize that chemosensory neurons may coordinate muscle excitability with other physiological relevant responses. Specifically, coordination of responses could be achieved through the release of neuroendocrine signals. Studies on the mammalian tubby gene, *Tub*, which is a highly conserved protein expressed in the nervous system, have already established a link between chemosensory function and metabolism. Mice with loss-of-function mutations in *Tub* suffer from late onset obesity and also display defects in ciliated neurons in the retina and inner ear [147-149].

Similarly, loss of *tub-1* function in *C. elegans* causes increased fat storage and defects in behaviors mediated by ciliated neurons [9,147,148,150]. These studies identified that lipid homeostasis is dependent on the neuroendocrine function of a set of 15 ciliated neurons, including the AWC neurons [9]. Consistent with this, I found that sensation of food can suppress the mobilization of fat stores in food-deprived animals. Interestingly, when I reduced the ability of chemosensory neurons to sense inedible *E. coli*, I saw a reduction in fat stores. This suggests that sensation of food by chemosensory neurons commonly block the effects of food deprivation on muscle excitability and fat mobilization. Additionally, I found insulin-like signaling, which is known to regulate lipid-homeostasis [82,116], regulates sex-muscle excitability.

An insulin-like signaling pathway integrates physiological and sensory-motor responses to food deprivation in *C. elegans*

Insulin-like signaling has been linked to many physiological responses associated with food availability, including life-span, fat homeostasis, and reproductive development [62,112-116,124]. In addition to regulating these long-term physiological changes, I have demonstrated that insulin-like signaling can also mediate acute changes in the excitability of sensory-motor circuits. Previous studies on *daf-2(lf)* mutants have demonstrated that majority of physiological defects caused by loss of insulin-like/DAF-2 signaling are dependent on the FOXO/DAF-16 transcription factor [117-119]. Interestingly, I have found that DAF-2-mediated changes in cell excitability in response

to food deprivation does not signal through the canonical FOXO/DAF-16, but instead via a novel pathway that includes PLC-γ, CaMKII, and EAG-like K^+ channels (Figure 18).

Historically, insulin signaling has been proposed to be active under well-fed conditions and regulate the body's ability to handle food by internalizing glucose. In *C. elegans*, a similar paradigm has been proposed with DAF-2/insulin-like signaling. Food deprivation has generally been proposed to inhibit DAF-2 activity, which can lead to FOXO/DAF-16-mediated dauer formation and increased longevity [112-116]. In contrast to studies in longevity and dauer formation, my data indicates DAF-2 activity is required under food-deprived conditions to suppress excitable motor output. This difference could possibly be attributed to sexually dimorphic differences, since males have been reported to have a significantly longer lifespan than hermaphrodites [151]. However, although DAF-2 is not required for increased male longevity compared to hermaphrodites, *daf-2(lf)* mutations still significantly increase male lifespan, suggesting that, insulin-like signaling also regulates similar pathways in males. Therefore, we propose that the assumption of food deprivation generally leading to decreased insulin-like activity in all tissues may not hold true in all biological contexts, and insulin-like signaling may be more complicated then originally thought under these conditions.

Interestingly, the requirement for active insulin-like signaling under food-deprived conditions has been investigated by groups studying other biological responses to food availability. For example, one characterized response to food deprivation, termed *F*asting-*I*nduced *R*edistribution of *E*sterase (FIRE), which results in the relocation of

intestinal esterase activity from the cytoplasm to the nucleus during fasting, has been reported to require DAF-2 activity [152]. Loss-of-function mutations in *daf-2* suppress the FIRE response associated with food deprivation. Additionally, DAF-2 activity is required for salt chemotaxis learning, which allows worms that have previously been exposed to NaCl under starved conditions negatively chemotax away from a NaCl source [39]. Similarly, *C. elegans* also associate temperature with food deprived conditions by avoiding temperatures at which they were previously food deprived. This behavioral association of food and temperature requires the insulin-like ligand, *ins-1*, which appears to negatively regulate DAF-2 activity in this context [38]. The observation that *ins-1* is required to actively suppress DAF-2 activity under food-deprived conditions suggest that DAF-2 can be active under these conditions and must be inactivated for certain biological responses. Interestingly, *ins-1* is thought to act as a DAF-2 agonist for salt chemotaxis learning. Thus, the action of DAF-2 and insulin-like signals under different environmental conditions is likely more complex than a general activation or reduction in DAF-2 activity, and likely varies depending on the physiological response in question. This could possibly explain the requirement for so many insulin-like ligands [153] and the observations that certain DAF-2 responses such as reduction in sex-muscle output and salt-chemotaxis learning [39] do not require the FOXO/DAF-16 transcription factor.

The finding that insulin-like receptor signaling can regulate acute changes in cell excitability in addition to metabolic and developmental responses to environmental conditions suggests that insulin-like peptides are commonly utilized to coordinate

multiple responses. We show that chemosensory neurons that have cilia exposed to the environment initiate changes in muscle excitability, suggesting these neurons likely modulate the release of insulin-like peptides. Interestingly, these same neurons also modulate the mobilization of fat stores and food exploratory behavior [9,95,100,146]. In regard to lipid metabolism, previous studies suggest that neuroendocrine regulation of fat storage is independent of DAF-2, since FOXO/DAF-16 mutants have no effect on *tub-1*-increases in fat storage [9]. Therefore, neuroendocrine regulation of muscle excitability and fat storage may be via different downstream effectors, or commonly through a FOXO/DAF-16-independent insulin-like signaling mechanism.

Is the coordination of metabolic and behavioral responses to food by insulin-like signaling conserved in other organisms?

Although the exact downstream effectors may differ, I propose that insulin/IGF-1 signaling also coordinates metabolic and behavioral responses in mammals. Consistently, food restriction in rodents results in increases in life span [12-14] and stress resistance [11,15,16], as well as a reduction in epileptic seizure susceptibility [10,17,18]. Interestingly, insulin-like growth factor I (IGF-1) signaling has been linked to life span and resistance to oxidative stress [30,154]. There are also several lines of evidence that suggest insulin signaling can regulate behavior as well. First, two areas of the vertebrate brain that contain the highest concentration of insulin receptors are the hypothalamus, which is involved in controlling animal activity, food intake, emotional

behavior, and sexual behavior, and the hippocampus, which is thought to regulate learning and memory [33,34]. Second, insulin has been linked to the ability of rats to learn the location of a submerged platform, and is known to increase memory in patients with Alzheimer's, suggesting that insulin can act on neuronal circuits in vertebrates as well [155-158]. Finally, similar to my findings in *C. elegans*, insulin administration in rats has been shown to have antiseizure activity in different behavioral seizure models [159].

In addition to the correlation between caloric restriction and insulin-like signaling in rodents, there is also data that indicates a role for CaMKII in insulin/IGF-I stimulation of glucose uptake by skeletal muscles [160]. Using *C. elegans* seizure susceptibility as a model, our work suggests that a similar insulin-like/CaMKII pathway may also reduce muscle excitability. Our data suggests that ligand binding to the insulin-like receptor on the muscles leads to Ca^{2+} activation of CaMKII through PLC-γ. CaMKII then phosphorylates and increases the activity of EAG K^+ channels, and the resulting efflux of K^+ ions causes decreased excitability (Figure 18). We propose that the regulation of muscle excitability by insulin-like signaling likely indicates a general mechanism used by organisms to regulate multiple behavioral and physiological responses to changing environmental conditions.

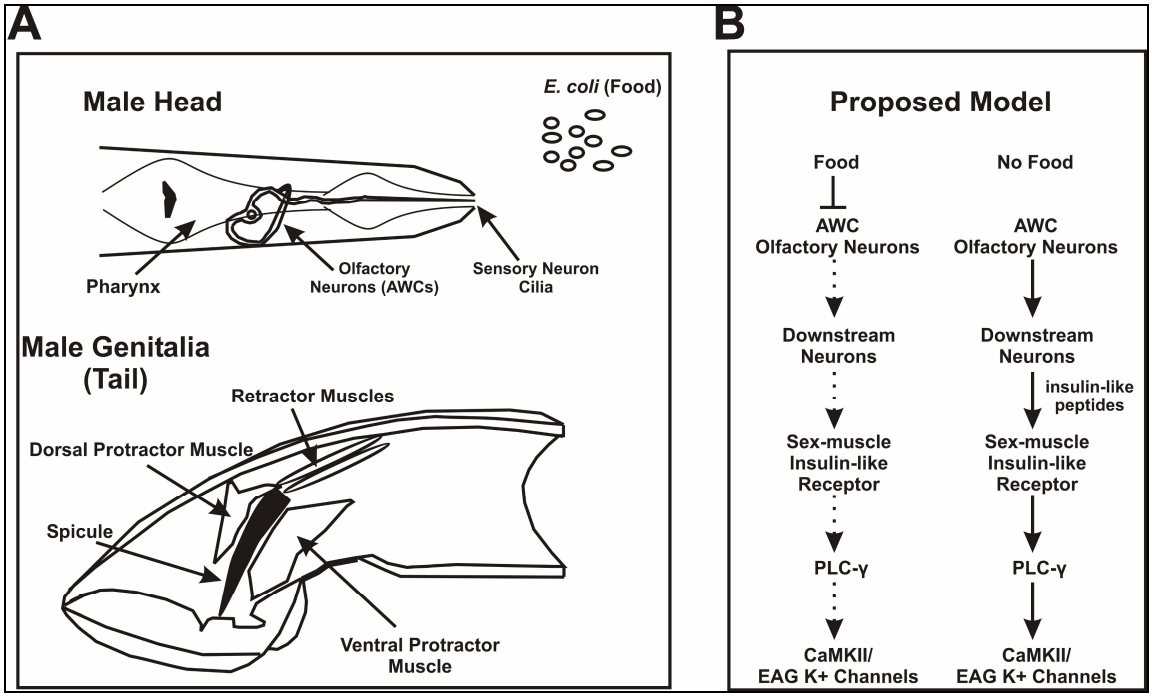

Figure 18. Chemosenory and insulin-like signaling regulate male sex-muscle excitability.
(A) Cartoon displaying the relevant structures in the head and male tail involved in chemosensory response to food deprivation. When food odors are present, AWC activity is attenuated.
(B) Proposed model for sensory regulation of sex-muscle excitability during food-deprivation. Under food-deprived conditions, AWC activity is up-regulated and results in release of insulin-like peptides from downstream neurons. Insulin-like peptides activate insulin-like receptors on the male tail, resulting in activation of PLC-γ and CaMKII. CaMKII then reduces sex-muscle excitability through the activation of EAG K^+ channels.

Future directions

The goal of my dissertation was to understand the cellular and molecular basis for how behavioral circuits are modulated by the environment. This study demonstrated that an organism can use sensory cues in the environment, independent of nourishment levels to adjust behavioral and physiological responses accordingly. I identified two mechanisms at which *C. elegans* males can determine nutrient availability, one is via

changes in the pharyngeal muscles and associated NSM neurons, and the other is via the activity of the chemosensory AWC neurons. These changes in pharyngeal and chemosensory neuron activity eventually lead to the observed decrease mutant-induced seizures in food-deprived males, and are also a possible mechanism for decreased mating efficiency. My results indicate both pharyngeal and chemosensory mechanisms converge by acting on the insulin-like receptor, DAF-2, in the sex muscles. Ligand binding to DAF-2 consequently leads to the activation of K^+ channels through the downstream effectors of PLC-γ and CaMKII. In order to gain a more complete understanding of how this motivated behavior is influenced by the environment, two remaining questions will need to be addressed: (1) determining the cellular mechanisms and neurotransmitters used by food-sensing circuits to communicate to the genitalia, and (2) revealing how the molecules downstream of DAF-2 regulate the electrophysiology of the sex muscles.

How do food sensing circuits communicate to the male genitalia?

At the cellular level, my work suggests that multiple tissues can sense decreases in nutrient availability and commonly regulate muscle physiology through an insulin-receptor/DAF-2-mediated mechanism. However, it is not clear how pharyngeal/NSM signaling or chemosensory/AWC signaling lead to activation of DAF-2. In *C. elegans*, more than 40 insulin-like ligands have been identified, including both agonists and antagonists of the DAF-2 receptor, many with an unknown expression pattern [145]. It is therefore possible that the NSM and AWC neurons act through a common or different

insulin-like peptide. It would be interesting to determine the ligand used in response to each of these mechanisms, and how these neurons communicate to the genitalia, whether by secreting it directly, or through activating other downstream neurons. One way to identify the ligands would be to do a screen in the *lev-11(rg1); unc-103(lf)* background and isolate mutant males that are no longer suppressed by *lev-11(rg1)*. These mutants could then be re-screened to focus only on those mutations that are also not suppressed by food deprivation. This screen should generate alleles in the downstream molecules, including *daf-2*, *plc-3*, *unc-43*, and *egl-2*, but these should be easily identified with complementation tests. Additionally, another way to identify the ligands would be to carry out RNAi on the known insulin-like ligands, and determine which insulin-like ligand is required for food-deprivation suppression of *unc-103(lf)*-induced seizures.

The identification that the NSM and AWC neurons can regulate sex-muscle excitability raises another interesting question about how single neurons can regulate relatively diverse behavioral responses. My work suggests that the NSMs can differentially regulate both the pharyngeal and sex muscles. Similarly, in addition to my finding that the AWCs regulate sex muscle excitability, others have shown that it also regulates chemotaxis to hermaphrodite phremones [103]. Both the NSMs and AWCs express more than one kind of neuropeptide/neurotransmitter [72,111,143], suggesting that secretion of multiple peptides may achieve the polymodal outputs of these neurons. Interestingly, the coexistence of multiple neuropeptides and neurotransmitters in a single neuron is also found in the mammalian nervous system [161]. This has resulted in much investigation into how neurons with multiple transmitters regulate their release: i.e. do

these neurons co-transmit multiple peptides or regulate the release of multiple peptides? There appears to be data in support of both mechanisms occurring, where regulated release is accomplished through storage in different sub-cellular compartments [161]. Nonetheless, the necessity of so many messenger substances in single neurons in the vertebrate nervous system is poorly understood. Here, it is possible that studying how pharyngeal and chemosenory neurons communicate to the genitalia could aid in this understanding. It would be interesting to determine if these neurons use regulated release of specific peptides or release a cocktail of peptides to control their diverse physiological outputs.

Although both pharyngeal and chemosensory neurons appear to influence sex-muscle excitability via the insulin-like/DAF-2 receptor, it is still possible that there are other neuropeptide receptors that regulate muscle excitability. The *daf-2(lf)* mutants analyzed were still partially suppressed by food deprivation. This could be due to the fact that these alleles are not null alleles, and still retain partial function, or that there are other mechanisms that do not signal through DAF-2. Since *daf-2* deletion alleles are lethal, determining if there are other pathways that can suppress sex-muscle excitability in response to food deprivation will have to be done by identifying other potential receptors.

One way to identify other pathways that can regulate muscle excitability in response to food conditions would be to determine the role of mechanosensation. In addition to chemosensation, it is likely that mechanosensation of the presence or absence of bacteria can also influence the mating circuit. Interestingly, previous research has

demonstrated that food-deprived worms will show an “enhanced slowing response” when they encounter bacteria, and this response requires dopamine-secreting neurons. The dopamine-expressing CEP, ADE, and PDE neurons are thought to regulate enhanced slowing response via mechanosensation of bacteria, since this response can be triggered by sterile Sephadex beads that are to large to ingest. Therefore, in contrast to chemosensory regulation of sex-muscle excitability when no food is present, mechanosensory neurons may suppress mating when a new food source has been located until the male is satiated. Consistent with this idea, I have preliminary data that suggests that mechanosensory and dopamine-expressing neurons are required for suppression of *unc-103(lf)* mutants in inedible food conditions (Appendix C). Identifying the neurons and downstream signals involved could lead to a better understanding of the complex mechanisms an organism uses to alter its behavior in changing environmental conditions.

Although caloric restriction has many beneficial effects on physiology that could possibly be exploited for health treatments, food deprivation also initiates food-scavenging behaviors that, if successful, will repress these adaptive responses. Therefore, in addition to understanding the mechanisms involved in activating these adaptive physiological responses, it will also be important to understand the mechanisms that repress them once food is located and ingested. Such experiments would include determining the length of time required for a food-deprived male to become food-satiated, and determining if the effect on mating is an active signal or simply the result of decreased pharyngeal, chemosensory, and insulin-like signaling.

To better understand how these signaling pathways increase or decrease sex-muscle excitability depending on the environmental condition, we will need to know how sex-muscle function is integrated with the other steps in male mating. Specifically, our data suggests that depriving males of food likely affects most steps of male mating, including contact response to hermaphrodites and vulva location behavior. Currently, our hypothesis is that food deprivation causes a general suppression of all mating steps, possibly by a common insulin-like signal. However, based on electron micrographs, there is extensive connectivity and crosstalk between the neuronal circuits that regulate mating, suggesting these specific sensory-motor outputs are integrated. Therefore, using the mating efficiency assay as opposed to spicule protraction mutants will be more informative here. Additionally, it would be interesting if other mating steps, such as staying at the vulva are actually influenced by sex-muscle activity. To determine this, it will be necessary to quantify the effects that food deprivation has on other mating steps, and determine if these effects are dependent on the voltage state of other mating tissues (i.e. the spicule muscles). Here, G-CaMP could be useful to monitor the activity of different tissues during each behavioral sub-step and possibly elucidate their temporal and functional relationships.

How do components of the insulin-like signaling pathway specifically control membrane excitability?

At the molecular level, I identified components of an insulin-like signaling pathway that can reduce muscle excitability and restore control of spontaneous seizures. Insulin is more widely known and studied for its role in glucose homeostasis and metabolism, however evidence that insulin can hyperpolarize vertebrate skeletal muscles were first reported over 50 years ago [162]. The ability of insulin to modulate neuronal and muscular function and is not as well known or studied, since its effect on membrane excitability is apparently not required for insulin stimulated glucose transport, which is important in studies on obesity and diabetes [163-165]. More recently, insulin administration in rats has been shown to have antiseizure activity in different behavioral seizure models [159], similar to my observations with *C. elegans* mutant-induced sex-muscle seizures. Because most of the ongoing work is focused on insulin's control of glucose homeostasis, not much is known about the exact mechanism of the antiseziure activity or its biological significance in non-epileptic animals. Here, insulin-like receptor/DAF-2-mediated reduction in mutant induced sex-muscle seizures could be used to further address these questions.

My data suggest that insulin-receptor-mediated changes in cell excitability are behaviorally relevant, possibly allowing insulin to commonly regulate metabolism and sensory-motor output under specific conditions. It has been reported in rodent models that decreases in cell excitability by insulin could be due to increased activity of Na-K

ATPase pump, and/or opening of inwardly rectifying K^+ channels that exist in skeletal muscle [165,166]. The requirement for the Na-K ATPase pump is not surprising and is likely not just specific to insulin-induced hyperpolarizations, since it is required to set up and maintain resting membrane potential [167-170]. Instead, my data suggests that EAG K^+ channels can function downstream of insulin signaling to hyperpolarize muscle cells. An important question remaining is how activation of EAG-like K^+ channels (EGL-2) in the sex muscles leads to decreased motor response during food-deprived conditions. For example, does activation of this channel just generally reduce membrane voltage of the entire muscle, or does it specifically affect the activation of certain Ca^{2+} channels, i.e. ryanodine receptor or L-type voltage-geated Ca^{2+} channels, by acting in the same temporal location? This type of question could be addressed by using the fluorescent Ca^{2+} indicator, G-CaMP, to measure Ca^{2+} in the sex muscles of wild-type and mutant males under different conditions. Additionally, to determine if insulin regulation of the muscles is spatiotemporal, it would be tremendously helpful to develop optical imaging techniques for using voltage-sensitive dyes in *C. elegans*. These techniques may be available soon, since other labs, such Li Lab (University of Texas, Southwestern Medical Center) are currently developing caged fluorophores and voltage-sensitive dyes to be used in *C. elegans*.

The finding that insulin-like receptor signaling leads to decreased excitability by Ca^{2+} activation of CaMKII via PLC-γ raises another interesting question: how can Ca^{2+} signaling regulate seemingly opposite cellular outputs? Specifically, Ca^{2+} is well known for its role in activating muscle contraction, and here, I provide evidence that it is also

involved in suppressing muscle output. How a single type of ion can regulate these different cellular states is not entirely intuitive. One possibility is that PLC-γ only leads to slight, local increases of Ca^{2+}, which is enough to activate calmodulin and CaMKII but not enough to induce myosin-actin cross-bridge cycling. This could then lead to the activation of downstream channels that reduce excitability and prevent the larger influxes of Ca^{2+} that lead to muscle contraction. Interestingly, studies on insulin-mediated glucose transport in rodent muscles have found that insulin results in a slight, but significant increase in Ca^{2+} concentration that is localized to the plasma membrane, and does not result in global changes in Ca^{2+} concentration [166]. Additionally, pharmaceutical drugs that specifically block CaMKII or PLC-γ also block insulin stimulated glucose transport [160,171]. Thus, it appears that insulin-mediated changes in glucose transport and cell excitability by insulin signaling are tightly integrated. Since the same signaling molecules appear to be conserved in both *C. elegans* and vertebrates to regulate muscle physiology, the regulation of *C. elegans* sex-muscle excitability by insulin-like signaling could be used to further dissect these physiological responses. For example, it will be important to identify the Ca^{2+} channels involved in local/slight increases in Ca^{2+} under food-deprived conditions.

Conclusion

C. elegans is a powerful model for studying the cellular and genetic properties that regulate neuromuscular circuits that control behavior. Its genetic tractability and

neuroanatomy permit analyses of cell activity within specific behavioral circuits. Since *C. elegans* contain many of the same proteins that govern cell-excitability in vertebrates, it is likely that mechanisms uncovered in this system will be relevant to developing strategies in higher organisms. Additionally, at the cellular level, *C. elegans* also appear to integrate complex sensory-motor circuits and physiological responses to changing environmental conditions. These observations underscore the utility of using this model to study how neural circuits are integrated to control different behavioral states. In addition to elucidating medically important modulators of physiology, further analysis and development of *in vivo* techniques to study the integration of *C.elegans* male mating and feeding behaviors could eventually lead to understanding the molecular details governing behavioral choice and motivation.

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APPENDIX A

SUPPLEMENTARY FIGURES AND TABLES

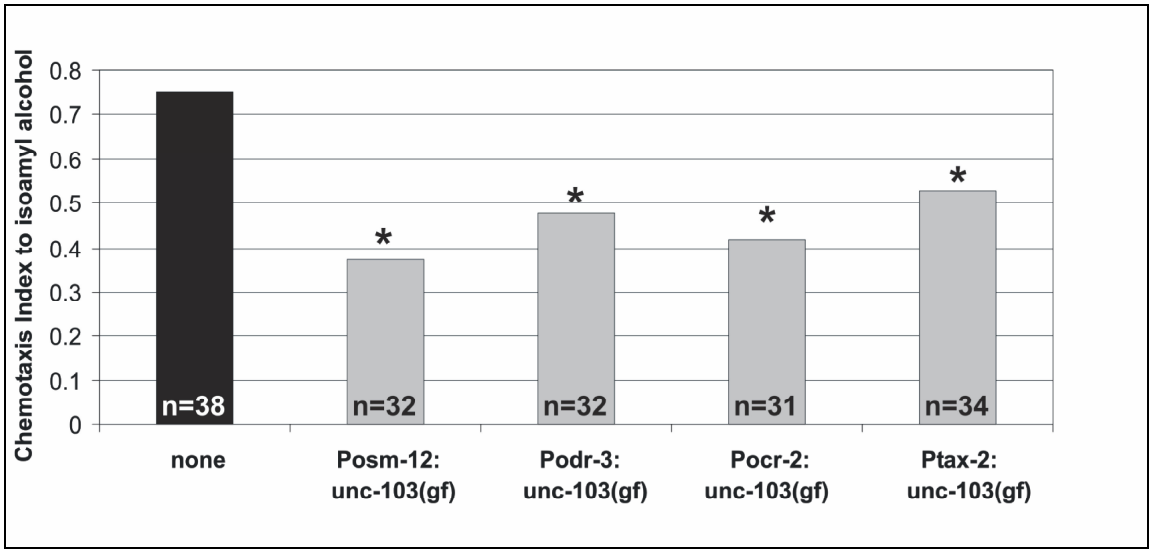

Figure A-1. Chemosensory-*unc-103(gf)* constructs cause reduced chemotaxis to isoamyl alcohol. Males were placed on an NGM plate in the middle of two areas containing 1µl of isoamyl alcohol and 1µl of 1M sodium azide on one side of the plate and 1µl of water and 1µl of 1M sodium azide placed at the other end of the plate. Chemotaxis index was determined based on the number of animals at the attractant (isoamyl alcohol) and transgenic animals were compared to wild-type controls. The * indicates a significant difference from non-transgenic controls (p-value < 0.05, Fisher's Exact Test).

unc-103 promoter sizes and expression pattern

Table A-1. Promoter size and expression pattern for *unc-103* 1st exons

Exon that is expressed	Size of upstream region	Expression Pattern
1A	2.5 Kb	body-wall muscles, intestinal muscle, anal depressor muscle, all sex-muscles, 4 neurons in the head
1B	5.8 Kb	Neurons (ADL, AVG, PVT, PVD, URA, URB, CEP, PHA, PHB, PVC, PCA, PCB, SPD, SPV), ventral cord neurons, ray neurons 1-9, sphincter muscle, spicule muscles
1C	2.7 Kb	DVA, PVT, GLR, TIA, SIBD, AVK, RIG, VD ventral cord neurons, pharyngeal muscle, ray neurons 1,3,4 and 9
1D	3.2 Kb	URA, PVM, ray neurons 1-9
1E	5.4 Kb	AIB, RIM, AIY, AVJ, ALA, AVH, NSM, I5 anal depressor muscle, all sex muscles
1F	2.2 Kb	ALA, ADL, ASK

Primers used in this dissertation

Table A-2: Primers used for *lev-11* , *unc-103* , and *tph-1* expression, and for TnT RNAi		
Target		**Primer sequence reading from 5' to 3'**
unc-103race2		CCTGGATCCGACACCACCTGACACGC
unc-103race5		GGGGACAAGTTTGTACAAAAAAGCAGGCTGGCCACGCGTCGACTAGTAC
unc-103race6		GGGGACCACTTTGTACAAGAAAGCTGGGTCGACAATTTCCAGTGG
tnt-1 , exon 5		GCTAAGGGAGGAAAGGATGTCATGAGCAAGG
		GTCATAGTCTTGACGTTCACGTCTCTTCTCC
tnt-2 , exon 5		AAAGAACCTTGAACCACCAAAGTACGT
		AGCTCCCTCATTTTGGCTTTGAACTGG
tnt-3 , exon 5		GCAGCCAAACGCCGTCACGAGGAAG
		TTCTTCTTGACGACGACGGTCATCAGCAGCACGA
tnt-4 , exon 3		CGGAGAAGCTCTAAATGAAGGTGAG
		CGCTCGGCAGCTTCACGCTCTTCTTGC
T7 promoter sequence*		TTAATACGACTCACTATAGGGAGA
unc-103 promoter		GGATCAGAATCACGTCATGCTGCAATCCAATTAGC
		TGCCACGTGGTTGTGTG-TGTGTACCC
unc-103 promoter & coding		GCGCGCTAGCGGATCAGAATCACGTCATGCTGCAATCCAATTAGC
		GCGCGCTAGCTAGAATAGTATCAGTTTCTTGTGTGG
tnt-4 promoter & coding		GTTCTCATAAACCTGACGGCGCTCCTT
		CCAAAATTAGCTCAATTGATCAAATAACTG
tph-1 promoter		GGTTTTCACACAGCTTTGGGTATTC
		TGCGGATGCCATCTGAAACAACG
tph-1 promoter, attB flanked		GGGGACAAGTTTGTACAAAAAAGCAGGC TTCGGTTTTCACACAGCTTTGGGTATTC
		GGGACCACTTTGTACAAGAAAGCTGGGTCTGCGGATGCCATCTGAAACAACG
lev-11 bodywall/sex muscle promoter		CACAGCGATGATGTGTCATGGCTTCC
		GCCTGCATCTTCTTCTTAATGGCGTCCAT
lev-11 pharyngeal/intestine promoter		ACTAGTCACTTGGCTTCACCCAGATGGG
		CAACCATGGCGAGCTTACGGGC
lev-11 promoter and coding(12kb)		CACAGCGATGATGTGTCATGGCTTCC
		CAGGACTGTTGATATTGC-ATGGCCCTGAA
lev-11 promoter to exon 3a	(Primer 1)	CACAGCGATGATGTGTCATGGCTTCC
	(Primer 2)	GGAGAAGGAGAAGACCGTTCAAGAGGCTGAGGCTGAGGTCGCTTCTTTGA
lev-11 exon 4b to 3'UTR	(Primer 3)	CAGGACTGTTGATATTGCATGGCCCTGAA
	(Primer 4)	TCAAAGAAGCGACCTCAGCCTCAGCCTCTTGAACGGTCTTCTCCTTCTCC
lev-11 pharyngeal promoter to 3'UTR		ACTAGTCACTTGGCTTCACCCAGATGGG
		CAGGACTGTTGATATTGCATGGCCCTGAA
*This sequence was added to the 5' of the *tnt* primers for dsRNA production.		

Table A-3. Primers used for chemosensory promoters, PLC RNAi, PLC-3 expression, LGG expression, and *egl-2 rescues*	
Primer Name	**Sequence**
Attb1daf2	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAATATTGTCAGATGTCG
Attb2daf2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGACAAGTGGATGATGCTC
AttB1Pocr2	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAGTACTTCGACTACTGGATGTAAG
AttB2Pocr2	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTAATGATGTGATGTACTCTACTG
AttB1Podr3	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCAAGACCAATTAGCAACTCGCTCC
AttB2Podr3	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTATGAGTAATTGATTTGAAATATCG
Attb1PO12	GGGGACAAGTTTGTACAAAAAAGCAGGCTGTACTACACGTGGACAAGCG
RAttb2PO12	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTTTACAGTGGAAGCTGAG
Attb1Ptax2	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGATCGGTTGACAATCAGTAGC
Attb2Ptax2	ACCACTTTGTACAAGAAAGCTGGGTCATCGGAAAACTCCGGTTTTTCTGAC
ATTP1	GACGTTGTAA AACGACGGCC AGTCTTAAGC
ATTP2	GGCCAGAGCT GCCAGGAAAC AGCTATGACC
FT7plc2	TTAATACGACTCACTATAGGGAGAGCAACCAACTGGTCTACGC
RT7plc2	TTAATACGACTCACTATAGGGAGACCACAACCTCCATTTCTCTC
FT7plc3	TTAATACGACTCACTATAGGGAGATAGATTGCTGGGATGGAC
RT7plc3	TTAATACGACTCACTATAGGGAGAACTCATCAGTTTTCACCAC
Attb1Pplc3	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGGTCCTCATCAGTACCCCACCACCAG
Attb2Pplc3	GGGGACCACTTTGTACAAGAAAGCTGGGTCAGACGACGATGATGGGCCAAGTGAGC
HIIIYFP	GGGAAGCTTATGAGTAAAGGAGAAGAACTTTTCACTGGA
NRLGGYFP	GTTGTTCTCCTCCTTGTAAGCCCACTTCATTTTGTATAGTTCATCCATGCCATGTGTAAT
NATTB2LGG	ATTACACATGGCATGGATGAACTATACAAAATGAAGTGGGCTTACAAGGAGGAGAACAAC
XBALGG	GGGTCTAGAGAAGTGATAGTCTATGACTAGAAAGCGAGAC
ATTB1UPSEGL2	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGTGGAAGGCGCATGCAGC
ATTB2EGL2DWN	GGGGACCACTTTGTACAAGAAAGCTGGGTATTGTTAGATTTTTCCGGC
pstegl-2atg	GGCCCTGCAGATGCCGGTTGGGAAACGTGGACTTGTGGC
kpnegl2tga	GGGGTACCCCTCATATCCGTGTTCTTGTCGGAGGACGGGC

APPENDIX B

IDENTIFYING DOWNSTREAM EFFECTORS OF NSM SIGNALING

Genetic screen to identify downstream effectors of NSM signaling

My data suggests that there are at least two redundant mechanisms that suppress sex-muscle excitability in response to low food availability, one via the pharyngeal NSM neurons, and one via the AWC chemosensory neurons. Both of these processes appear to converge by acting on the insulin-like receptor/DAF-2. However, the insulin-like neuropeptides used by these neurons to communicate to the genitalia are unknown. The tropomyosin mutation, *lev-11(rg1)*, and RNAi knockdown of pharyngeal troponin T (*tnt-4*) suppresses the abnormal sex muscle output of *unc-103(lf)* by altering pharyngeal muscle activity and NSM activity. To identify molecules downstream of NSM signaling, I designed a sensitized screen to identify alleles that can restore spontaneous seizures in the *lev-11(rg1)* background. I constructed the strain *lev-11(rg1)*; *unc-103(sy557)*; *him-8(e1489)*; *tnt-4(gk136)/+*, *unc-60(m35)/+* to carry out the sensitized screen. The *him-8(e1489)* mutation results in an increased frequency of X chromosome nondisjunction, and therefore increased frequency of males. *tnt-4(gk136)* is a deletion mutant that is lethal when homozygous; *unc-60(m35)* lies in close proximity to *tnt-4* on chromosome five and is used as a balancer. *unc-60(m35)* homozygous animals are extremely uncoordinated in movement, while heterozygotes move normally and are easily recognized. The *lev-11* and *tnt-4* alleles suppress the abnormal sex muscle output of *unc-*

103(lf) by acting in the pharyngeal muscles, and Prc mutants isolated in this background should identify genes that work in the pharyngeal-mediated pathway or in an alternate pathway to inhibit sex muscle contraction.

Young adult *lev-11(rg1)*; *unc-103(sy557)*; *him-8(e1489)*; *tnt-4(gk136)/+, unc-60(m35)/+* hermaphrodites were soaked in EMS and allowed to self-fertilize. I picked 750 F1 hermaphrodites to individual plates and allowed them to self-fertilize, and kept plates that contained 25% or greater F2 Prc males. From 1500 mutagenized gametes, 37 lines were isolated that appeared to have increased pentrances of spontaneous spicule protraction.

Table B-1: Spicule protraction mutants isolated in *lev-11(rg1)* background

strain	% Prc (n)	St. Dev	Additional Phenotypes
control	16 (139)	0.067	na
rg392	50 (40)	0.212	sluggish, insertion defective
rg396	44 (36)	0.114	?
rg400	36 (58)	0.033	contact response and turning defective
rg404	35 (91)	0.045	?
rg405	63 (43)	0.115	pharyngeal pumping during mating
rg412	33 (49)	0.009	?
rg414	64 (55)	0.032	?
rg418	81 (27)	0.017	?

To determine which mutants to analyze first, I re-screened all 37 lines for spontaneous protraction and additional mating defects. Of the 37 potential mutant lines, I identified 8 lines that appeared to significantly restore spontaneous muscle seizures. Table B-1 displays eight alleles that are possible candidates of molecules involved in pharyngeal-sex muscle integration. The control in Table B-1 refers to the original strain,

lev-11(rg1); *unc-103(sy557)*; *him-8(e1489)*; *tnt-4(gk136)/+, unc-60(m35)/+*. One of the 8 alleles, *rg405*, had an interesting phenotype that suggested it affected NSM function. During mating, my data suggests that the NSM neurons repress pharyngeal pumping; however, I found that *rg405* was deficient in this response. I therefore choose this allele to analyze first.

Preliminary behavioral and mapping data of *rg405*

rg405 restores spontaneous spicule protraction of *unc-103(dn)* in the *lev-11(rg1)*; *unc-103(sy557)*; *him-8(e1489)*; *tnt-4(gk136)/+, unc-60(m35)/+* background. To better understand how this allele disrupts the function of pharyngeal/NSM function, I crossed out the other mutations, and outcrossed *rg405* to wild type five times. I then measured the effects of food deprivation and specific mutations previously shown to affect spicule protaction mutants (Table B-2). Interestingly, food deprivation slightly, but significantly, suppressed *rg405*-induced sex-muscle seizures, suggesting that this allele is not affecting all food-deprivation mechanisms. Additionally, *rg405* requires UNC-64/syntaxin, which is necessary for synaptic vesicle release. Combined with the observation that *rg405* does not reduce NSM-mediated pharyngeal contractions during mating, my data suggests that *rg405* may be affecting NSM-mediated signaling.

The *rg405* allele also seems to have an interesting interaction with the ERG-like K^+ channel, UNC-103 (Table B-2). I found that combining *rg405* with the *unc-103(dn)* allele results in nearly 100% of males suffering spontaneous seizures, while combining

rg405 with *unc-103(0)* resulted in no synergistic affect. Although the mechanism behind this effect is not clear, one possible hypothesis is that the protein defined by *rg405* interacts with UNC-103 to regulate cell excitability. In the *unc-103(dn)* background, the interfering properties of *unc-103(dn)* may result in a more severe dysfunction of the *rg405* or *unc-103* proteins.

Table B-2: Preliminary genetic interactions with *rg405*

Genotype	% Spicule-protracted males (n)
lev-11(rg1); unc-103(dn); tnt-4/+; rg405	63 (43)
rg405	54 (100)
rg405 STARVED	30* (30)
lev-11(rg1); rg405	47 (49)
unc-64(e246) ; *rg405*	15* (55)
unc-103(0)	33 (42)
unc-103(lf)	73 (49)
unc-103(0); rg405	31* (45)
unc-103(lf); rg405	95* (55)
rg405; T01B4 cosmid	23* (47)

*p-value < 0.05, Fisher's exact test

To identify the gene affected by *rg405*, I used single nucleotide polymorphism (SNP) [172] and linkage mapping to originally map the lesion to the right end of LG X. I then used more recombinant lines to further map *rg405* to a region between Segment 19 and Segment 20 (about a 1Mb region) of LG X. I then injected cosmids containing candidate genes in this region to determine which, if any, could rescue *rg405*-induced spicule protraction. Interestingly, I found that the T01B4 cosmid, which only contains the genes *twk-21* and *twk-22*, appeared to significantly reduce *rg405*-spontaneous muscle seizures (Table B-2). I sequenced *twk-22* and found no mutation in *twk-22*, however,

sequencing data for *twk-21* was inconclusive. It is possible that *rg405* may define *twk-21*, however, more sequencing will have to be done to verify this. Alternatively, since the injection of the cosmid did not completely rescue *rg405*, it is possible that overexpression of these tandem pore K^+ channels may be the reason for suppression, and not due to *rg405* affecting these genes. Therefore, if no mutation is found, more detailed recombinant mapping will need to be completed to clone *rg405*.

APPENDIX C

MECHANOSENSORY NEURONS REGULATE SEX-MUSCLE EXCITABILITY IN RESPONSE TO FOOD CHANGES

MEC-10 and TRP-4 expressing neurons suppress sex-muscle excitability

I have previously shown that inedible food can partially block the effects of food deprivation on suppression of sex-muscle excitability. This effect, in part, is due to the suppression of chemosensory neuron activity. In addition to effecting chemosensory neurons, inedible bacteria should also have an affect on mechanosensory neurons. This is supported by the finding that chemical attractants do not mimic the inedible food condition unless they are combined with Sephadex beads (data not shown). To determine if mechanosensation of the presence or absence of food in the environment also influences sex-muscle excitability, I paired *unc-103(lf)* males with known mechanosensory loss-of-function mutants (Tables C-1 and C-2). The *mec-10* gene encodes an amiloride-sensitive Na^+ channel required to sense gentle touch stimuli along the body [173], and the *trp-4* gene encodes a worm homolog of *Drosphila* and zebrafish transient receptor potential channel (TRPN1) proteins needed for mechanotransduction by sensory epithelia [174-176].

Table C-1. Mechanosensory mutations influence sex-muscle excitability

Condition	Genotype	% Spicule-protracted males (n)
Food	*unc-103(0)*	34 (59)
	mec-10(e1515)	3 (37)
	unc-103(0); mec-10(e1515)	52 *(96)
	unc-103(dn)	75 (40)
	unc-103(dn); mec-10(e1515)	100 (43)
Inedible Food	*unc-103(0)*	17* (58)
	unc-103(0); mec-10(e1515)	29 (21)
	unc-103(dn)	48 *(31)
	unc-103(dn); mec-10(e1515)	71 (21)
No Food	*unc-103(0)*	8 (25)
	unc-103(0); mec-10(e1515)	0 (30)
	unc-103(dn)	11 (35)
	unc-103(dn); mec-10(e1515)	22 (18)

*p-value < 0.05, Fisher's exact test

Interestingly, I found that the *mec-10(e1515)* mutation significantly increased the percentage of males with sex-muscle seizures on both food and inedible-food conditions, and had no effect on the no-food condition (Table C-1). Thus, in contrast to chemosensory neurons, mechansensory neuron activity is not required to suppress sex-muscle excitability when no food stimulus is present. However, the increased percentages seen when food stimulus is present, suggests that *mec-10*-expressing neurons are required to suppress sex-muscle excitability when a food-deprived male first encounters a food source. Therefore, it is possible that these neurons may integrate with pathways that turn off DAF-2-mediated reductions in sex-muscle output. This will have to be tested more thoroughly and the specific neurons involved will need to be identified. *mec-10* is expressed in the touch-receptor neurons (ALM, AVM, PVM, PLM) and FLP and PVD neurons.

Table C-2. *trp-4* expressing neurons suppress sex-muscle excitability

Condition	Genotype	% Spicule-protracted males (n)
Food	*unc-103(dn)*	75 (40)
	unc-103(dn); trp-4(sy695)	58 (92)
Inedible Food	*unc-103(dn)*	48 *(31)
	unc-103(dn); trp-4(sy695)	54 (28)
No Food	*unc-103(dn)*	11* (35)
	unc-103(dn); trp-4(sy695)	39 (23)

*p-value < 0.05, Fisher's exact test

In contrast to the *mec-10(e1515)* mutation, I found that *trp-4(sy695)* [176] resulted in increased *unc-103*-induced muscle seizures on both inedible-food and no-food conditions (Table C-2). These results suggest that TRP-4-expressing mechanosensory neurons suppress sex-muscle excitability when the animal is food deprived. However, similar to the experiments with *mec-10(e1515)*, this assumes that the reason these animals have increased sex-muscle excitability is due to general mechansensory neuron dysfunction in *trp-4*-neurons, and not the *trp-4(sy695)* allele itself. This can be further tested by determining the site of action of *trp-4(sy695)* and ablating the corresponding neuron(s).

trp-4 is expressed in the dopamine-secreting neurons (CEP, ADE, DVA, DVC, and PDE), suggesting that these neurons may use dopamine to communicate to the genitalia. As a preliminary experiment to test this hypothesis, I tested the effect of dopamine exposure on sex-muscle response to the ACh agonist, arecoline. Interestingly, I found that males pre-exposed to a 30mM dopamine plate for 30 minutes had a reduced sensitivity to arecoline-induced spicule protraction (Table C-3).

Table C-3. Sex muscle response to Ach agonist, arecoline	
Condition	**% males that protact spicules in 1mM arecoline**
Wild type	97 (31)
Wild type, pre-exposed to dopamine	59* (29)

*p-value < 0.0001

Since my preliminary results suggest that mechanosensory neurons may reduce sex-muscle excitability by secreting dopamine, I analyzed dopamine receptor mutants to see if I could phenocopy the effects of *trp-4(sy695).* Interestingly, I found that the deletion *dop-2(vs105)* allele (*dop-2(0)*) significantly increased the percentage of food-deprived males with seized muscles, similar to the *trp-4(sy695)* mutation (Table C-4). Therefore, it is possible that in addition to insulin-like signaling, dopamine signaling also regulates the excitability of the male genitalia under non-optimal conditions. It will be interesting to determine whether DOP-2 acts in the sex muscles like DAF-2, or if it acts elsewhere, such as the genital neurons to suppress muscle excitability.

Table C-4. DOP-2 is required for food-deprivation suppression of *unc-103(lf)*		
Genotype	**% Well-fed males protracted (n)**	**% Food-deprived males protracted (n)**
unc-103(0)	39 (56)	7 (55)
unc-103(dn)	56 (16)	11 (35)
unc-103(0); dop-2(0)	51 (80)	34* (61)
unc-103(dn); dop-2(0)	65 (23)	41* (17)

*p-value < 0.05, Fisher's exact test

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