CIRCUITS ATTENUATING SEIZURES UNDER WELL-FED AND FOOD-DEPRIVED CONDITIONS IN *C. elegans* MALE SEX MUSCLES

A Dissertation

by

BRIGITTE LEBOEUF

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

May 2009

Major Subject: Biology

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Approved by:

Chair of Committee,	L. Rene Garcia
Committee Members,	Sumana Datta
	Arne Lekven
	Mark Zoran
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ABSTRACT

Circuits Attenuating Seizures Under Well-Fed and Food-Deprived Conditions in *C. elegans* Male Sex Muscles. (May 2009)

Brigitte LeBoeuf, B.S., University of Dallas

Chair of Advisory Committee: Dr. L. Rene Garcia

The circuits that allow organisms to control behavioral timing need to be tightly regulated to ensure execution of appropriate environmental responses. Disrupting such regulation results in individuals unable to perform tasks necessary for survival and propagation. Identifying the molecular components regulating behaviors will enable compensation where behavioral impediments to survival exist. To identify circuits of behavioral regulation, I studied male mating behavior in the nematode *Caenorhabditis elegans*. Specifically, I focused on the step wherein the male inserts his copulatory spicules into the hermaphrodite vulva, as vulva penetration is required for successful sperm transfer. This step must be tightly regulated; if the spicules protract too soon or not at all, vulva penetration and thus successful mating will not occur.

In this dissertation, I elucidate the circuits regulating sex-muscle excitability under standard conditions and describe how these pathways are augmented to further reduce excitability under food deprivation conditions. I employ a variety of assays to identify and analyze these circuits, including genetic manipulation, biochemical techniques, and behavioral assays. Under standard conditions the calcium/calmodulin-

dependent protein kinase II (CaMKII) encoded by *unc-43* is required to inhibit *C. elegans* male sex-muscle seizures; under conditions where food is scarce, I propose that CaMKII is further up-regulated to activate the EAG K+ channel EGL-2 through a direct interaction. The CaMKII/EGL-2 interaction functions to attenuate calcium influx from L-type voltage-sensitive calcium channels (L-VGCCs), while CaMKII also down-regulates calcium influx from ryanodine receptors. Additionally, another K+ channel, the voltage- and calcium-sensitive big current channel SLO-1, attenuates sex-muscle excitability by inhibiting L-VGCCs under food deprivation conditions. In conclusion, CaMKII and EGL-2's paralog, UNC-103/ERG-like K+ channel, are required when food is plentiful to prevent premature sex-muscle contractions, while food deprivation reduces cell excitability and thereby inhibits inappropriate seizures through CaMKII, EGL-2, and SLO-1.

DEDICATION

This work is dedicated to Matante Pauline and Ti John and all those who have gone before me. May your love always light my way.

ACKNOWLEDGEMENTS

This work would not have been possible, and God knows would not have been completed, without the help and guidance of many people, especially my advisor, Dr. L. Rene Garcia. My committee members, Drs. Sumana Datta, Arne Lekven, and Mark Zoran provided valuable insights to further my research career. The colleagues I have worked with on this journey have been truly wonderful. Dr. Daisy Gualberto is an excellent lab manager and an even better friend. Her tireless effort and support served to make this project easier, and her stories and discussion made the long days in lab more entertaining. My fellow graduate students Dr. Todd Gruninger and Yishi Liu provided discussion, analysis, and support when I needed it most.

I would not get anywhere without the constant love and support of my wonderful family. My parents, Albert and Jacqueline LeBoeuf, offered their encouragement in many ways, and my sisters, Nicole and Lindsay, were always there for me. The time I spent working on this dissertation saw many changes in our lives, especially those that affected my beloved cousins, John and Audra, most acutely. In particular, the birth of John and his wife Kristina's daughters Maya Elizabeth and Kate Pauline, Audra and her husband Christopher's daughters Greta Bertha and Anya Karren, as well as the deaths of our grandparents, Albert and Bertha LeBoeuf and their parents, John and Pauline Pontes. I am sorry their parents and our grandparents are not here to see me finish this project, but I am eternally grateful that Greta, Maya, Anya, and Kate are here to light up our lives.

My grandparents Margaret and Harry Hedge kept me entertained and though my grandfather is not here to see me finish this project I am grateful for the guidance he provided. In addition, I always enjoyed talking with my uncles Jeff and Mike, particularly about sports. Such conversations offered much-needed breaks from the daily grind of research.

I would especially like to thank Dr. Veronica Giselle Martinez Acosta, who traveled this path before me and always offered excellent advice, and her husband Alfonso, who didn't mind my frequent intrusions. My friends Jodi Bollinger, Robin Young, Catherine Brand, and Rachel Russo have always provided much-needed support and distractions for which I am eternally grateful.

Lastly, I would like to thank the only individuals who cared if I came home at night: my cats Shanahan, Kennedy, and Indy. Shanahan has been with me from the beginning, and abhors the long hours I spend in the lab. Kennedy was with me for only a short time but was a great friend to Shanahan. Indy is the newest addition to the family and is always ready to offer some love and affection.

NOMENCLATURE

ACh	acetylcholine
ARE	arecoline, an acetylcholine receptor agonist
CaMKII	calcium/calmodulin-dependent protein kinase II
CFP	cyan fluorescent protein
EAG	*ether-a-go-go* K+ channel
egl	egg-laying defective
EGL-2	*C. elegans* homologue of EAG K+ channel
EGL-19	*C. elegans* homologue of L-type Ca^{2+} channel
ERG	*ether-a-go-go* K+ channel related gene
gf	gain of function
GST	glutathione s-transferase
LEV	levamisole, an acetylcholine receptor agonist
lf	loss of function
MBP	maltose binding protein
RyR	ryanodine receptor
unc	uncoordinated
UNC-43	*C. elegans* homologue of CaMKII
UNC-68	*C. elegans* homologue of RyR
UNC-103	*C. elegans* homologue of ERG K+ channel
YFP	yellow fluorescent protein

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CHAPTER I

INTRODUCTION

Understanding how behaviors are regulated at the molecular level

All animals must regulate the timing and order of responses within a complex behavior to accomplish specific tasks or react to environmental changes. When responses are incorrectly regulated, the ability to respond to specific situations is lost. For instance, a person suffering from schizophrenia perceives stimuli similar to a healthy person, but the disease causes them to display spontaneous emotional outbursts that interfere with their ability to respond appropriately to the stimuli. Drugs used to suppress the symptoms of schizophrenia occasionally allow afflicted individuals to react correctly, but these drugs do not treat the cause of the disease, and they also produce many other deleterious side effects [1]. By determining how behaviors are regulated, better ways to modify inappropriate behavior can be developed. To accomplish this, the genetic and molecular components that control specific behaviors need to be identified, and how these components work together under the appropriate conditions needs to be understood.

I am interested in studying how behavioral regulation is accomplished at the molecular level. This involves identifying the genes responsible for regulating a set of responses as well as where gene expression is necessary and what other molecules the

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This dissertation follows the style and format of PLoS Genetics.

gene product interacts with. Although studying behavior in vertebrates is feasible, humans and laboratory model animals such as mice and zebrafish contain complex neuronal systems composed of billions of cells, thus rendering thorough analysis a daunting task. The more complex an organism, the more difficult it is to determine what occurs at the level of cells and molecules. To learn how a multicellular organism coordinates behavioral responses at the molecular level I chose to study the nematode *Caenorhabditis elegans*.

Advantages provided by *Caenorhabditis elegans* for studying behavioral regulation

The advantages offered by *C. elegans* in discovering the intricate apparatus that controls behavior include intrinsic attributes of the animal and a multitude of tools available to exploit the organism's instructional potential. *C. elegans* is a 1 mm long hermaphroditic round worm commonly found in vegetation. It is an appealing laboratory animal since large quantities of the worm are easy to cultivate and isolates can be stored in glycerol at -80°C or in liquid nitrogen to remove the necessity for perpetual propagation of lines. In addition, *C. elegans* is a powerful genetic system becaise the entire genome has been sequenced and is highly annotated, it has a short generation cycle in which it matures from an egg to adult in three days, hermaphrodites lay a large number of progeny, and many mutations result in easy-to-follow morphological and behavioral phenotypes. Originally exploited by Sydney Brenner for

studying how the nervous system develops and functions, the entire lineage of each of the somatic cells in an adult hermaphrodite is known [2-4]. In addition, wiring projects have identified much of the connectivity that exists between the 302 neurons in the hermaphrodite and 381 neurons in the male, as well as their innervation of other cell types [5,6](http://www.wormatlas.org). Since the worm is transparent under the microscope, individual cells can be identified and removed, facilitating the discovery of their roles in behavior. Additionally, due to the relative ease of creating transgenic lines, genes can be manipulated to determine the nature of their function in particular cells. These attributes of *C. elegans* make it a powerful and appealing organism in which to study what occurs in cells at the molecular level and how that affects the overall function of the animal.

The advantages of *C. elegans* as a model organism to advance the realm of scientific knowledge has recently been acknowledged by the Nobel Prize committee, as three Nobel Prizes have been awarded for research conducted on *C. elegans* in the past six years: Sydney Brenner, Robert Horvitz, and John Sulston received the 2002 Nobel Prize in Medicine for the establishment of *C. elegans* as an organism for molecular and cellular studies, specifically the identification of proteins involved in programmed cell death [3,4,7]; Andrew Fire and Craig Mello received the 2006 Nobel Prize in Medicine for the discovery of RNA interference (RNAi), a post-transcriptional regulation of messenger RNA [8]; and Martin Chalfie received the 2008 Nobel Prize in Chemistry for developing the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* as a biological marker [9].

Many scientists have taken advantage of *C. elegans* to address biological questions, including those involving neuromolecular studies of behavior. These studies have included all the basic tasks an organism needs to accomplish for survival: locomotion, sensing the environment, feeding, defecation, and reproduction. Studying how these behaviors are regulated has revealed general principles of neuronal signaling pathways as well as how specificity is obtained in regulating behavior.

Excitable cells control behavior

Behavioral output is achieved through signaling events that occur in excitable cells, specifically neurons and muscles. Channels and pumps maintain the charge separation that distinguishes excitable cells. More negatively charge ions are on the inside of a cell while more positively charge ions are on the outside of a cell. Neuronal signaling occurs when channels open to allow positively charged ions to flood the cell, neutralizing the negative charge inside the cell and depolarizing the membrane. The sudden reversal of membrane polarization in a neuronal cell is referred to as the action potential. This electrical signal can be propagated along the length of the neuron, and be passed to other neurons through both electrical and chemical signals as well as muscle cells using chemical signals.

Muscle contraction is initiated by motor neurons that release a chemical signal, called a neurotransmitter, onto muscle cells. Neurotransmitters open channels that depolarize the membrane. In turn, the membrane depolarization opens calcium

channels. The calcium then causes muscle contraction by allowing myosin and actin to interact, shortening the muscle fibers. In a relaxed muscle, myosin and actin are prevented from interacting by tropomyosin, a protein that blocks the myosin binding sites in actin. Calcium then binds the tropomyosin regulatory complex troponin, which moves tropomyosin off the actin/myosin interaction site, allowing binding to take place and the muscle fiber to shorten [10,11].

Maintenance of membrane polarization and a return to membrane resting potential after an excitation event is control by K^+ channels. K^+ channels open in response to membrane depolarization and allow positively charge ions to flow out of the cell, shutting off voltage-dependent Ca^{2+} channels. Pumps then return Na^+ and Ca^{2+} ions to the outside of the cell while K^+ ions are returned to the inside of the cell. The removal of Ca^{2+} allows the muscle cell to relax. Disrupting the function of these channels and pumps results in the inability of organisms to properly regulate behavior. The excitable cells can be too excitable and respond under inappropriate conditions or not excitable enough and stay dormant when they should respond to a stimulatory signal. The study of molecular regulation in *C. elegans* has helped identify specific roles for channels in coordinating behavior [12].

Locomotion

C. elegans moves as a result of body bends created by relaxation of body wall muscles on one side and contraction of body wall muscles on the other. These bends

result in a sinusoidal wave that propels the animals forward or backward [13]. The wave is produced by cholinergic neurons that stimulate muscle contraction on one side of the animal and inhibitory GABAnergic neurons that induce muscle relaxation on the other side [14]. Mutations affecting *C. elegans* locomotion are easily identified by sight and were the first type of behavior mutants generated by Sydney Brenner [4]. Brenner classified worms displaying movement defects as "uncoordinated" or "Unc," and these defects ranged from complete paralysis to jerky movements. Analysis of the genes defined by the Unc phenotypes has facilitated the discovery of their roles in processes from development to behavior, including the initial discovery of genes involved in neuronal signaling pathways [15]. Genes encoding UNC-13, UNC-18, and UNC-64 were identified to be important for synapse-located neurotransmitter release [16,17]. Since these proteins are conserved in all metazoans (in vertebrates, UNC-13 and UNC-18 have the same name but are distinguished by adding "M" in front, and UNC-64 encodes syntaxin), studying these genes in *C. elegans* facilitated the analysis of how general synaptic transmission is controlled [18]. However, mutations that affect locomotion often affect other behaviors, including feeding, defecation, chemosensation, and mating, indicating that such genes have a broad impact on behavior. While studying locomotion gives insight into general principles of neuronal regulation, studying other behaviors allows for determination of how specific regulation is obtained.

Chemosensation

An organism needs to be able to respond to environmental stimuli, to either move towards positive signals that indicate food or mates, or away from noxious stimuli that indicate harmful situations. The neurons, receptors, intracellular signal transduction pathways, and regulatory mechanisms have been identified in *C. elegans* that allow them to respond to many chemical stimuli [19]. In *C. elegans,* a subset of sensory neurons mediates attraction while another subset mediates repulsion [20]. The chemosensory neurons ASH extend processes to the front of the worm that are exposed to the environment and are involved in the avoidance response to a large range of noxious stimuli [21]. This pair of neurons releases glutamate onto the AVA and AVD command interneurons responsible for activating motor neurons that initiate backward movement [6,22]. Environmental stimuli are transduced into the cell through G-protein coupled receptors located in the ASH neurons; these receptors include *sra-6* and *srb-6* and they could be attached to the G-proteins defined by *odr-3* and *gpa-3*, which are involved in sensing noxious stimuli [23]. Heterotrimeric G-proteins are ubiquitous second messengers that upon activation split into two subunits, Gα and Gβγ, that regulate a variety of processes. ASH function can be modulated by the $G\alpha_i$-like protein *gpa-11*, which increases ASH response when food is plentiful [24]. How the ASH neurons work in responding to noxious chemicals is one example of how studying chemosensation in *C. elegans* has identified the molecular basis of how specificity is obtained in regulating behaviors.

Feeding

The study of feeding behavior, controlled by a muscular pump called the pharynx, allows for understanding of how behavioral modifications can be integrated throughout the animal at both a cellular and molecular level. The pharynx is responsible for ingesting and grinding up bacteria by an intrinsic contraction rhythm before passing the food on to the intestines. Twenty neurons innervate the pharynx, but successful feeding is dependent upon only one neuron, M4, to move food from front to back in the pharynx [25,26]. Roles for the other nineteen neurons have been discovered by looking at how pumping efficiency is modulated. One such neuron, the NSM, has no effect on pumping under standard conditions; when the neuron is removed via laser ablation the pharynx works normally [26,27]. However, a role for the NSM in how the worm coordinates feeding with male sexual behavior has been discovered [28]. When mating, a male's pharynx stops pumping; disrupting the NSM allows the males to feed and mate at the same time [28,29]. The NSM neuron appears to play an additional role in signaling to the male genitalia, as removing the neuron increases sex muscle excitability [28]. Loss of function in a ERG-like K+ channel (*unc-103*) responsible for reducing cell excitability is hypothesized to caused hypersensitivity of the NSM neuron, as removing the *unc-103* gene results in pumping while mating [28]. Thus, this neuron is involved in the integration of the feeding state of the animal with mating.

Defecation

C. elegans defecation is a rhythmic behavior that occurs every 45 seconds, with a series of coordinated abdominal muscle contractions that first compress the gut contents and then expel them [30,31]. Perturbation of the defecation cycle through mutations allows for studying how rhythms are maintained in *C. elegans*. Mutating the inositol triphosphate receptor (IP3 receptor) *itr-1* can result in complete disruption of the defecation cycle [32]. IP3 receptors allow bursts of calcium from the endoplasmic reticulum into the cytoplasm of the intestinal cells, resulting in a calcium wave that travels from posterior to anterior every 45 seconds [32-34]. Functional ITR-1 receptors, along with other proteins involved in regulating the defecation cycle, only need to be present in intestinal cells, and not neurons, to control defecation, suggesting a role for non-neuronal somatic cells in regulating behaviors [32,35]. While the intestinal calcium wave regulates the first part of defecation, compression of the gut contents mediated by a signal from two GABAnergic neurons, AVL and DVB, controls expulsion of the gut contents from the cloaca [31,36]. Coordination of compression and expulsion occurs via a signal secreted from the intestines, using proteins similar to those involved in neurotransmitter release [37]. Exploration of the regulators of defecation has identified molecules involved in controlling a rhythmic behavior. In addition, it has provided insight into intercellular signaling utilized by non-neuronal cells.

Egg-laying

Similar to feeding and defecation, egg-laying is a rhythmic behavior dependent upon the function of a small number of cells. Egg-laying is not necessary for propagation, as eggs retained in the hermaphrodite hatch and the progeny eat their way out of the adult. The HSN neuron innervates the muscles that contract to open the vulva and allow eggs to leave [3,6]. Egg-laying is stimulated by the neurotransmitter serotonin, but its role is not straightforward [38]. Thus, analyzing the effects of serotonin on egg-laying has allowed researchers to analyze how one molecule can both positively and negatively regulate a behavior. Serotonin receptors are present on both the vulva muscles and the HSN neuron itself, allowing the neurotransmitter to act in an autocrine manner. While serotonin activates the vulva muscles through $G\alpha_q$, a member of the heterotrimeric G-protein family, it inhibits the HSN neuron through the activation of a different G-protein, $G\alpha_o$ [39,40]. So far, five different serotonin receptors have been identified in the egg-laying circuit: SER-1, SER-7, and SER-5 in the muscle and SER-4 and MOD-1 in the neuron [41-44]. The muscle-located serotonin receptors activate G-proteins that induce muscle contraction, while in the neuron SER-4 activates the inhibitory G-protein $G\alpha_o$ and MOD-1 encodes a unique Cl- ion channel [41,45]. Lack of SER-4 or MOD-1 leads to over-stimulation of the vulva muscles and results in hermaphrodites laying eggs at an earlier stage than normal, while loss of SER-1, SER-7, and SER-5 or their associated G-proteins inhibits egg-laying [41-45]. The study of serotonin's role in the egg-laying system has revealed how neurotransmitters can control

behavior in a context-specific manner, as serotonin can activate and inhibit the muscle contractions leading to egg-laying.

Male mating behavior

While *C. elegans* is predominantly a hermaphroditic species, males can appear in populations through nondisjunction of the X chromosome, leading to a sex that has its own specific behavior: mating. Hermaphrodites, containing both sperm and eggs, display no mating behaviors; wild-type hermaphrodites will even move away from interested males [46]. Males, on the other hand, cannot pass on their genetic code without mating, and therefore have developed specific structures and a complex behavior that allows them to inseminate mates. Males' tails consist of a fan-like structure that includes both a sensory apparatus and a copulatory complex consisting of neurons, muscles, and spicules (Figure 1) [5]. The male utilizes his spicules to penetrate the hermaphrodite vulva, and the muscles controlling the spicules, the protractors and retractors, are attached to its base. A nonessential anal depressor muscle is also electrically coupled to the protractors (http://www.wormatlas.org). Neurons involved include the SPC motorneuron, post cloaca sensilla, and hook sensilla [5]. The behavior these neurons coordinate can be broken down into stereotyped steps: contact, searching, vulva location, prodding, spicule insertion, and sperm transfer (Figure 2).

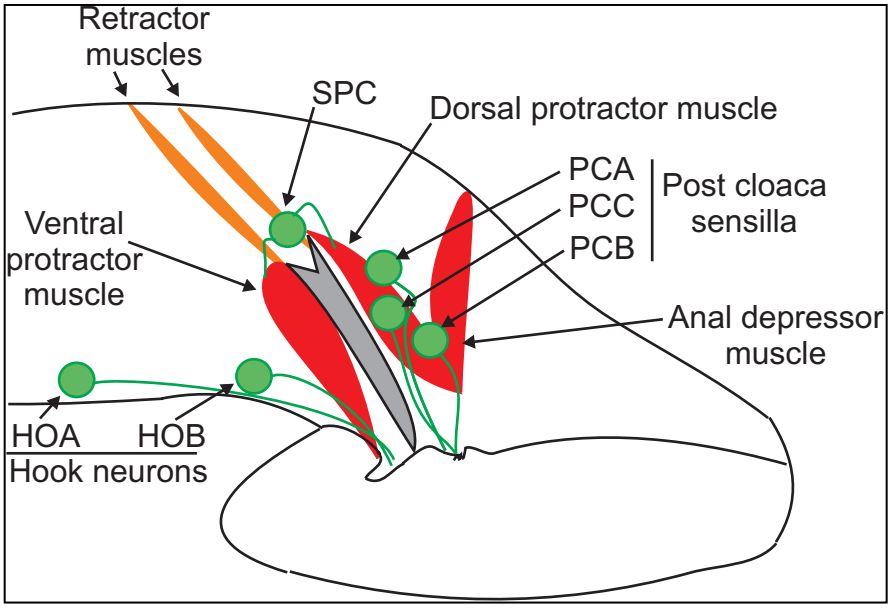

Figure 1. Diagram of the male tail. Spicule is in gray, neurons and their processes are in green, and muscles are in red and orange. The protractors and retractors attach at the base of the spicules, and the SPC neuron synapses to the protractors. The hook neurons (HOA and HOB) send processes to the front of the cloaca opening while the post cloaca sensilla (PCA, PCB, PCC) send processes to the area immediately behind the cloaca opening. The post cloaca sensilla share connectivity with one another as well as the SPC and protractor muscles.

Contact is initiated when the male locates a hermaphrodite [29]. He positions his tail along the hermaphrodite cuticle (Figure 2A) and initiates a backward movement, pressing the sensory structures in the male tail on the cuticle as he scans for the vulva (Figure 2B). If the vulva is not located on one side of the hermaphrodite, the male initiates a turn and scans the other side, stopping when he locates the vulva and positioning his spicules over the vulva slit (Figure 2C). To penetrate the tightly closed vulva, the male initiates a rapid, rhythmic prodding of the spicules (Figure 2D) [47]. Prodding is controlled by the protractor muscles attached to the base of the spicules and

initiated by the sensory hook neurons (HOA and HOB) and post cloaca sensilla (p.c.s.: PCA, PCB, PCC) (Figure 2D) [5,47]. The rapid muscle contractions are controlled by calcium released from ryanodine receptors (RyRs) located in the membrane of the endoplasmic reticulum [47]. Once the vulva slit has been penetrated, the protractor muscles tonically contract, causing the spicules to fully insert into the vulva (Figure 2E). Tonic contraction of the protractor muscles is initiated by the release of the neurotransmitter acetylcholine (ACh) from the SPC motor neuron (Figure 2E) [5]. Tonic spicule muscle contraction is dependent on L-type voltage-gated calcium channels (L-VGCCs) [47]. After the spicules are inserted into the vulva, sperm transfer occurs (Figure 2F) [14]. Upon completion of sperm transfer, the male retracts his spicules using the retractor muscles, attached at the base of the spicules, and moves away [5]. These individual steps can be dissected using genetic and molecular tools to determine how behaviors are controlled at the level of cells and molecules.

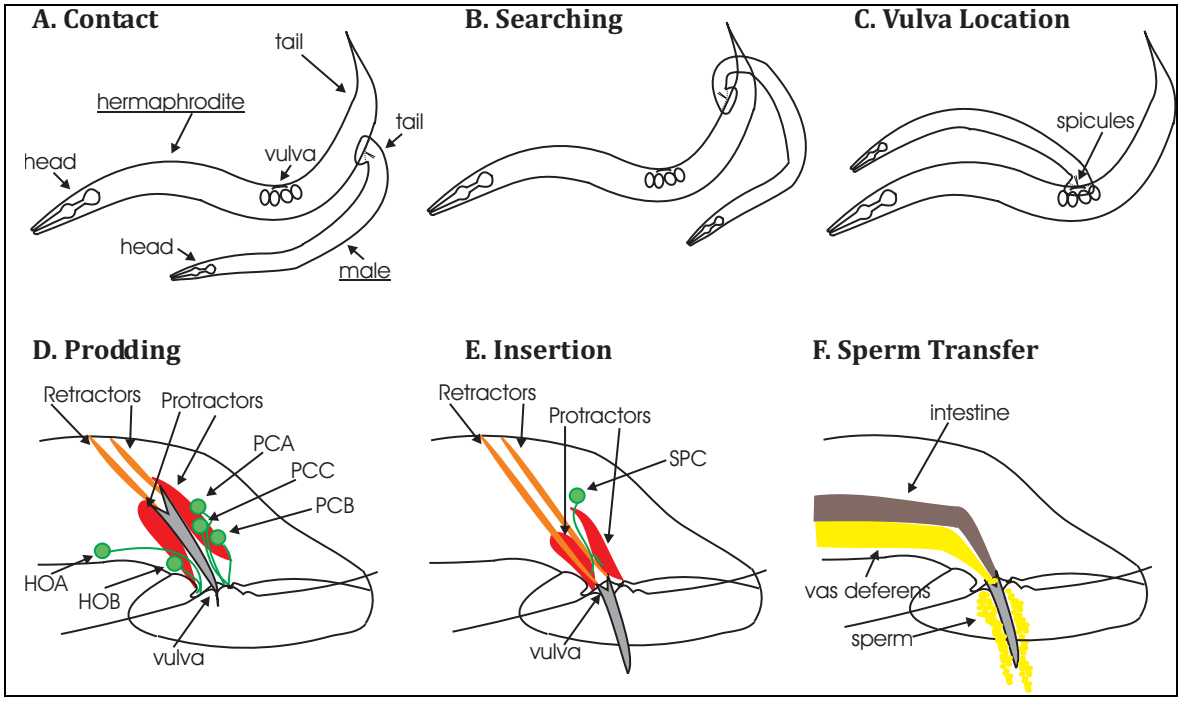

Figure 2. Steps of *C. elegans* male mating behavior. The spicule is in gray, the muscles in red and orange, and the neurons and their processes are in green. (A) The male initiates contact and positions his tail on the hermaphrodite cuticle. (B) The male presses his tail along the cuticle, scanning for the vulva. (C) The male stops moving and positions his spicules over the vulva slit. (D) Muscles and neurons involved in spicule prodding. (E) The protractor muscles contract, causing the spicules to fully insert into the vulva. Spicule protraction is controlled by the SPC motor neuron. (F) Full spicule insertion allows sperm transfer to occur.

Spicule insertion step of male mating behavior

In my exploration of how behaviors are controlled at the molecular level, I focused on the spicule insertion step of male mating behavior. *C. elegans* offers advantages for studying males: since male mutations can be propagated in hermaphrodites, there is no need for balanced strains when mutations result in males unable to mate. Additionally, mutations exist that increase the proportion of males in a

population; the mutation *him-5(e1490)* is used throughout this study and increases the percentage of males in a population from <0.01% to 33% [48]. Since the timing of spicule protraction has to be tightly regulated to occur only when the vulva slit has been penetrated, the spicule insertion step allows for the study of how sensing external cues is integrated with preprogrammed motor responses. To study the mechanisms involved in inhibiting spicule protraction, Dr. L. Rene Garcia isolated mutations that permanently protract their spicules in the absence of mating cues (Figure 3) [12]. Males displaying this mutant phenotype can be easily identified under a microscope, facilitating the analysis of mutants. Since the behavior has to be tightly regulated, it is possible to obtain mutations that cause spicule protraction but do not interfere with additional behaviors. I will focus on the genes defined by two alleles resulting in the mutant phenotype in this study: *sy557*, which causes 82% of homozygous males to protract their spicules, and *sy574*, which induces protraction in 56% of males (Table 1) [12,49].

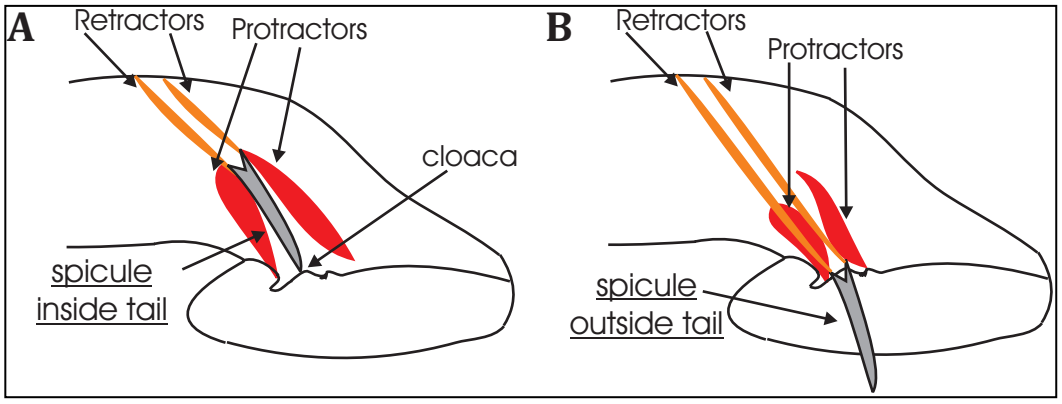

Figure 3. Permanent spicule protraction in the absence of mating cues. The spicules are in gray and the muscles are in red and orange. (A) Wild-type spicule position. (B) Mutant spicule position.

ERG-like K+ channel/*unc-103* regulates spicule insertion

Dr. L. Rene Garcia located the *sy557* molecular lesion to the gene *unc-103*, which encodes the *C. elegans* ortholog of the *ether-a-go-go* related gene (ERG) K+ channel. *unc-103(sy557)* induces spicule protraction in 82% of males, while hermaphrodites display no gross abnormalities (Table 1). A deletion allele, *n1213*, hereafter referred to as *unc-103(0)*, induces spicule protraction in 42% of males (Table 1). In addition to causing abnormal protraction, these mutations in *unc-103* result in premature protraction during prodding, making it more difficult for the males to insert their spicules into the vulva [12].

ERG K+ channels function to repolarize a cell membrane after a depolarization event has taken place [50]. Human ERG K+ channels (hERG) are expressed in the heart and mutations in hERG lead to cardiac arrhythmias that result in sudden death [51,52]. hERG not only helps with repolarization of the membrane but also prevents premature depolarization events from occurring, and in this way inhibits cardiac arrhythmias. Overcoming the potentially lethal results of interfering with hERG function would be of great benefit to organisms.

Since 58% of males lacking the ERG-like K+ channel *unc-103* do not display spontaneous spicule protraction, a compensatory mechanism is likely active in some males to inhibit these muscle seizures. To identify the compensatory mechanisms, I focused on the mutant allele *sy574*, identified in the same screen as *sy557*, that induces spicule protraction in 56% of males (Table 1) [49]. By identifying the location of the

sy574 lesion and determining its role in regulating the timing of spicule protraction, I hope to elucidate how molecules function together to coordinate behaviors.

Table 1. Mutations causing male spicule protraction		
Genotype[a]	**% Protracted (*n*)**	***p* value**[b]
Wild-type	12 (106)	
unc-103(sy557)	82 (55)	<0.0001
unc-103(0)	42 (91)	<0.0001
sy574	56 (300)	<0.0001
[a]Strains contain *him-5(e1490)* [b]Fisher's Exact Test to Wild-type		

Dissertation objectives

The objective of this dissertation is to elucidate how behaviors are controlled at the molecular level by identifying the genes involved in regulating the timing of spicule protraction in *C. elegans* males.

Chapter II provides detailed materials and methods used in experiments to obtain the dissertation objective. In Chapter III, I locate the *sy574* lesion to the gene CaMKII/*unc-43* and identify body-wall and sex muscles as the area of function for *unc-43* in controlling spicule protraction. In Chapter IV, I explore the relationship between *unc-43* and EAG K+ channel/*egl-2* in reducing seizures under food deprivation conditions. I discovered that UNC-43 can directly bind EGL-2 and this binding is dependent upon serine 567 on the EGL-2 c-terminus. In Chapter V, I describe the partially redundant mechanisms that reduce the effects of calcium influx from two

calcium channels, L-type voltage-gated Ca^{2+} channels (L-VGCCs) and ryanodine receptors (RyRs), under both well-fed and food deprivation conditions. EGL-2 and UNC-43 attenuate the effects of L-VGCC activity under food deprivation conditions while UNC-43 plays a further role in reducing the activity of RyRs. I also identified the importance of the BK K+ channel/SLO-1 in down-regulating male sex-muscle excitability under both food-satiated and food deprivation conditions. These circuits are in place to respond to different environmental conditions to ensure mating behavior is performed at the appropriate time.

CHAPTER II

EXPERIMENTAL PROCEDURES

Strains

All strains contain *him-5(e1490)* [48] and were maintained as described in [4]. The following strains were used. LGI: *lev-11(rg1)* [28]; LGIII: *unc-103(n1213)* [53]; *unc-103(sy557)* [12]; *pha-1(e2123)* [54] *unc-64(e246)* [4]; LGIV: *unc-43(sy574)* [49]; *unc-43(e408)* and *unc-43(e266)* [4]; *unc-43(n1186)*, *unc-43(n1179)*, and *unc-43(n498)* [53]; *unc-43(sa200)* [31]; *egl-19(n582)* [55]; LGV: *unc-68(r1158)* [56], *egl-2(n693)* [57], *egl-2(n904)* [58], *egl-2(rg4)* [49], and *slo-1(js379)* [59].

Identification of *sy574*

sy574 was isolated as described in [12]. *sy574* animals were out-crossed five times. Single nucleotide polymorphism mapping was used to locate the *sy574* lesion to a 570-kb region on Chromosome IV between cosmids R102 and K08F4 [60]. Candidate genes in the map region were PCR amplified from wild-type genomic DNA and injected into *sy574* hermaphrodites. *sy574* injected strains also contained the *pha-1(e2123)* mutation, which is a temperature sensitive allele that prohibits pharyngeal development at 20°C, resulting in death. A wild-type copy of *pha-1* on plasmid pBX1 was injected along with the PCR amplified wild-type genomic DNA. The genes that were amplified

are: *gpa-7*, R09H10.6, F13B12.3, C43F9.6, *tax-6*, and *cal-4*. The primer pairs used to amplify the genes are as follows: FCAL4 and CAL4R for *cal-4*, R09H106R and FR09H106 for R09H10.6, FC43F96 and C43F96R for C43F9.6, Nwgpa7f and Gpa7nwr for *gpa-7*, ff13b123 and F13B123R for F13B12.3, and Ftax6 and tax6r for *tax-6*. None of these genes rescued *sy574*-induced spicule protraction. In addition, complementation tests were done between *sy574* and alleles of *unc-43*, a gene too large to PCR amplify from the genomic DNA. 4 out of 5 *unc-43* alleles tested failed to complement *sy574*, indicating that the *sy574* lesion is in *unc-43*. The *unc-43* gene in *sy574* animals was sequenced and two missense changes were found: *sy574*A changes the sequence CACGGATTT to CACGAATTT, and *sy574*B changes GCCGCGTGT to GCCGTGTGT. *unc-43* was also sequenced in the PS1385 strain used for mutagenesis and no mutations were found.

Identification of the molecular lesion in *unc-43(e408)*

unc-43(e408) was identified as previously described in [4], but the molecular lesion has not been reported. I sequenced the *unc-43* gene from *unc-43(e408)* animals and found one missense change: TTGTCGCCA to TTGTTGCCA.

Generation of the *egl-2* deletion allele *rg4*

Trimethylpsoralen mutagenesis was applied to *egl-2(n693gf) him-5* animals to generate the *rg4* deletion in *egl-2*. *egl-2(n693gf)* causes hermaphrodites to retain eggs; to select for animals lacking *egl-2*, worms that displayed normal egg-laying behavior were kept. The progeny generated by these worms were then screened by PCR analysis with primers to internal *egl-2* exons, looking for worms that do not have *egl-2*. *egl-2(rg4)* contains exons 1-7 but does not include the pore region or the *egl-2(n693gf)* lesion [58]. The *egl-2(rg4)* deletion ends before the start of the next gene, *pme-5* [49]. The *egl-2(rg4)* deletion is 4023 base pairs long and the sequences flanking the deletion are: aagtgaactccattcacgatc and ttttgaaaaaaaattttcaaa (http://www.wormbase.org). *egl-2(rg4)* animals were out-crossed four times.

Identification of the molecular lesion in *egl-2(n904)*

egl-2(n904) was identified as previously described by [58], but the molecular lesion was not reported. We sequenced *egl-2* from *egl-2(n904)* worms and found the missense change GCATCTGAC is changed to GCATTTGACG.

Assay for abnormal spicule protraction

~20-30 virgin L4 males were isolated on NGM plates containing the *E. coli* strain OP50. The males were allowed to develop to adults overnight and were scored as positive for spicule protraction if at least one spicule partially extended from the cloaca.

Mating behavior assay

10 one-day-old adult *unc-64(e246)* hermaphrodites were placed on a 9 mm circle of one-day-old OP50. One male was added to the plate and once contact was initiated with a hermaphrodite mating was observed for 10 min or until the male transferred sperm. The steps of male mating including hermaphrodite contact, turning, vulva location, spicule insertion, and sperm transfer were recorded using the Microsoft Excel program reported in [61].

Male potency assay

L4 males were selected and allowed to mature overnight on plates with or without OP50. Paralyzed L4 *unc-64(e246)* hermaphrodites were isolated from males and grown for two days on plates containing OP50. Two-day-old hermaphrodites were placed one per plate on 10 mm NGM plates containing an OP50 lawn that was allowed to grow overnight. One male per plate was then placed with the hermaphrodites for 20

minutes at 20°C. After 20 minutes, the males were removed. The plates were scored two days later; a male was considered to have successfully mated a least one moving progeny was present on the plate.

Pharmacology

5-10 one-day-old virgin males raised overnight on plates with or without OP50 were placed in Pyrex, round-bottom, three-well titer plates in 500 μl of various concentrations of levamisole (LEV) (MP Biomedicals, http://www.mpbio.com) or arecoline (ARE) (Indofine Chemical Company, http://www.indofinechemical.com). The males were left in the drug concentration for 5 min and males that protracted at least one spicule for 10 sec were scored as positively reacting to the drug. The concentrations of LEV and ARE were prepared in sterilized ddH_20 from stock solutions of 1 mM and 1 M, respectively. Graph Pad Prism 5 software (Graph Pad Software, Inc., http://www.graphpad.com) was used to determine curve fits and the EC_{50} and EC_{90}.

Cell ablations

The protocol to laser ablate cells was followed as presented in [62]. Mid-L4 males were placed on 5% noble agar pads containing 4 mM NaN_3 to paralyze the worms. Cells were identified based on location and ablated using a Spectra-Physics (http://www.spectra-physics.com) VSL-337ND-S Nitrogen Laser attached to an

Olympus (http://www.olympusamerica.com) BX51 microscope. Non-ablated control males were also placed on the agarose pads containing 4 mM sodium azide. After ablation, males were placed on NGM plates containing OP50 overnight to recover.

Food deprivation assay

L4 males were transferred to NGM plates without OP50 and allowed to crawl away from the bacteria used to transfer them. The males were then picked by a mouth pipette containing M9 to a fresh plate lacking OP50. To keep the males from leaving, an 8 M glycerol ring was added along the outside of the plate. Males were scored the next day for the spicule protraction phenotype.

Plasmid construction

A list of primers and their sequences used in this study are provided in the Appendix (Table A-1), as is a list of plasmids (Table A-2).

Plasmids containing *unc-103* genomic DNA were created as described in [63].

Plasmids containing the *unc-43* cDNA were constructed as follows. Primers U43cDNAstart and U43cDNA3UTR were added to 3' RACE products made from mixed-stage populations of *him-5(e1490)* worms using BD SMART RACE cDNA amplification kit (BD Biosciences, http://www.bdbiosciences.com) to obtain *unc-43* cDNA. Plasmid pBL12b was created by cloning *unc-43* isoform g

(http://www.wormbase.org) into pBR322 [64] between restriction sites *Msc*I and *Eag*I. All plasmids containing DNA sequences generated by PCR were sequenced to ensure no mutations were induced. A point mutation was found in pBL12b that changes an amino acid and was corrected by single site mutagenesis using primers fpbl12a and pbl12ar to create plasmid pBL13. A yellow fluorescent protein (YFP) PCR fragment was amplified using primers FYFPEAG and YFPRKPN from the plasmid pSX95.77YFP (plasmid courtesy of N. Moghal, Huntsman Cancer Institute, University of Utah, Salt Lake City, UT), cut with *Eag*I and *Kpn*I, and ligated into the same sites in pBL13 to create plasmid pBL14. The Gateway Reading Frame Cassette A (Invitrogen, http://www.invitrogen.com/) was inserted into the *Eag*I site of pBL14 to create the destination vector pBL33. The Gateway recombination technology developed by Invitrogen allows for more efficient cloning by utilizing bacteriophage lambda recombination properties. Single site mutagenesis with primers Fpbl33ssm and Pbl33ssmr was used to remove YFP from pBL33 to create plasmid pBL33-YFP. Single site mutagenesis with primers Fpbl333utr and Rpbl33stop was used to remove the UNC-43 self-association domain from pBL33 to create plasmid pBL33-self asso.

Promoters were placed in front of the Gateway destination vectors pBL33-YFP and pBL33-self asso to drive tissue-specific expression. First, the promoters were PCR amplified from genomic DNA using primers containing Gateway ATTB sites. The PCR fragments were then placed in the Gateway entry vector pDG15 [63] using a BP recombination reaction. The *aex-3* promoter was amplified using primers attb1aex-3p and attb2aex-3p to create the plasmid pDG15aex-3 [65]. The *lev-11* promoter that

expresses in *C. elegans* body wall muscles was amplified using primers attb1lev11prof and attb2lev11pror to create plasmid pLR22 [66]. The *tnt-4* promoter was amplified using primers attb1tntproup and attb2tnt4prodown to create plasmid pLR25 [28]. The *acr-8* promoter was amplified using primers acr-8ATTB1 and acr-8ATTB2 to create plasmid pLR92. The *unc-103*E promoter was amplified to create plasmid pLR21 was previously described in [63]. An LR reaction was then performed between the promoter-containing plasmids and the destination vectors pBL33-YFP and pBL33-self asso to create plasmids capable of driving UNC-43 expression in various tissues. pDG15aex-3, pLR21, pLR22, pLR25, and pLR92 were recombined with pBL33-YFP to generate pBL70, pBL71, pBL69, pBL72 and pBL80, respectively. pLR22 and pLR21 were recombined with pBL33-self asso to generate pBL68 and pBL75, respectively.

The expression pattern of *acr-8* has not been reported previously. Plasmid pLR92 contains the *acr-8* promoter and this plasmid was recombined using an LR reaction with the YFP-containing Gateway destination vector pGW322YFP [63] to create plasmid pLR99.

To create the UNC-43 cDNA driven by a heat shock promoter, pPD49.78 was cut with *Eco*RV (plasmid pPD49.78 courtesy of A. Fire, Stanford University School of Medicine, Stanford, CA). The Gateway Reading Frame Cassette C.1 was cloned into this site, creating pTG14. *unc-43* cDNA was amplified from pBL33 using the primers unc43att2bcsf and unc43att2bc3r. The PCR fragment was then recombined with pDONR in a BP reaction to create pBL54. pBL54 was recombined with pTG14 in an LR reaction to generate pBL58.

We used the *gtl-1* promoter driving cyan fluorescent protein (CFP) in worm intestines as a marker to identify transgenic animals [67]. *gtl-1* was PCR amplified from genomic DNA using the primers att1gtl1p and gtl1patt2 and this PCR fragment was recombined with pDG15 in a BP reaction, creating Gateway entry vector pBL63. pBL63 was then recombined with the Gateway destination vector pGW77C using an LR reaction to create plasmid pBL66. pGW77C was created by cloning the Gateway Reading frame Cassette C.1 into a blunted *Xba*I site in the CFP-containing plasmid pSX95.77CFP (plasmid courtesy of N. Moghal).

Plasmids containing the *egl-2* cDNA were constructed as previously described in [49]. pTG44 contains wild-type *egl-2* cDNA expressed using the *unc-103*E promoter [63]. To generate *egl-2* cDNA with mutations, single site mutagenesis of pTG44 was performed using the following primer pairs: fegl2n698gf and Regl2698gf to create plasmid pBL111, containing the *egl-2(n693gf)* mutation; Fegl2n904 and Egl2n904r to create plasmid pBL109, containing the *egl-2(n904)* mutation; Fegl2rsvs and Egl2rsvsr to create plasmid pBL110, containing a mutation (S888W) in the potential CaMKII binding site RSVS; fEgl2end and egl2rsvsr to create plasmid pBL108, deleting aa 891-949 of EGL-2; and Fdelpasegl2 and Delpasegl2R to create plasmid pBL122, deleting aa 9-185 of EGL-2.

The plasmids used to express UNC-43 and the EGL-2 c-terminus in yeast cells for the yeast two-hybrid assay were created as follows. *unc-43i* was placed in the yeast expression vector pGBKT7 (Clontech Laboratories, Inc., http://www.clontech.com), tagged with a c-Myc epitope tag. pGBKT7 was cut with *Eco*RI and *unc-43* amplified

from pBL33 using primers func43iecor1 and unc43iecor1r was ligated to pGBKT7, creating plasmid pBL81. Single site mutagenesis was performed on pBL81 using the primers FUnc43inact and Unc43inactr, creating plasmid pBL85 that has the UNC-43 kinase domain inactivated by an D135N mutation. The UNC-43 inhibitory domain was removed from pBL85 by single site mutagenesis using primers Unc43irev and ForPGBKT7 to create pBL88.

The EAG K+ channel c-terminal domain was placed in the plasmid pGADT7 (Clontech), tagged with an HA epitope tag. *egl-2* was amplified from pTG44 using primers fegl2cterm and egl2cterm2r, cut with *Sac*I and *EcoR*I and ligated to pGADT7 cut with the same enzymes to create plasmid pBL93.

The EGL-2 K+ channel c-terminal domain was attached to maltose binding protein (MBP) by ligating PCR products into pMal-C2 (New England Biolabs, http://www.neb.com/nebecomm/default.asp?). *egl-2* was PCR amplified from pBL93 using primers fegl2cterm and egl2hind3r. After PCR amplification, the PCR product was cut with *EcoR*I and *Hind*III and ligated into pMal-C2 cut with the same enzymes, creating plasmid pBL99. Single site mutagenesis on pBL99 using primers Fegl2n904 and Egl2n904r created plasmid pBL114, which contains the *egl-2(n904)* point mutation.

The *unc-43* isoform lacking the self-association domain, *unc-43i* (http://www.wormbase.org), was attached to glutathione-s-transferase (GST) by ligating a PCR product into pGEX-3T. pGEX-3T was cut with *Sma*I and Gateway (Invitrogen) reading frame cassette RfC.1 was ligated to the plasmid, creating the destination vector pBL117. The entry clone pBL54 was created by amplifying *unc-43* cDNA from pBL33

using primers unc43att2bcsf and unc43att2bc3r and performing a BP reaction to place the PCR product in pDONR (Invitrogen). A LR reaction was then preformed using entry clone pBL54 and destination vector pBL117, creating plasmid pBL120, which contained the full-length *unc-43* isoform *unc-43g* attached to GST. To remove the *unc-43* self-association domain, single site mutagenesis was performed on pBL120 using primers Fpbl333utr and Rpbl33stop, creating plasmid pBL123.

Determining the expression pattern of *unc-43*

The *unc-43* promoter proved too large to clone into a plasmid. In order to determine the expression pattern of the gene, an 11-kb upstream region was amplified from wild-type genomic DNA using primers func43pro and u43prcfp. In addition, CFP was amplified from plasmid pGW77C using the primers cfpfu43p and u54rev. The PCR products from these two reactions were combined and a full-length *unc-43* promoter:CFP construct was obtained by amplification using primers func43pro and u54rev. The resulting *unc-43* promoter:CFP PCR product was injected along with the pBX1 plasmid containing *pha-1(+)* into *pha-1(e2123)* hermaphrodites following standard procedures [68]. Pictures of the expression pattern were taken with an Olympus FV1000 confocal microscope.

A second 5' UTR exists 9 kb upstream of the initial *unc-43* 5' UTR. To determine where expression is driven from this UTR, a 2.5-kb fragment upstream of the second 5' UTR was PCR amplified using primers U43att2bcsf and U43att2bc3r. This

short *unc-43* promoter was placed in pDG15 [63], resulting in plasmid pBL49. pBL49 was injected along with pBX1 into *pha-1(e2123)* hermaphrodites and progeny that survived were analyzed to determine the expression pattern of the short *unc-43* promoter.

Transgenics

Worms carrying transgenic lines were created as described in [68]. Worms expressing *unc-103* genomic DNA were created as described in [63].

To obtain worms with *unc-43* expressed on transgenic arrays, plasmids containing *unc-43* were injected into *unc-43(sy574);him-5(e1490)* or *unc-103(0);unc-43(e408);him-5(e1490)* hermaphrodites. All injection mixtures contained 20 ng/μl of pBL66. pBL66 contains the *gtl-1* promoter driving CFP in the intestines, allowing for the selection of transgenic lines. In addition, all injection mixtures contained pUC18 to obtain mixtures with the DNA concentration of 200 ng/μl. The *unc-43* plasmids were injected in the following concentrations: 50 ng/μl of pBL69, pBL72 and pBL70, 26 ng/μl of pBL58, and 10 ng/μl of pBL71. After injection, F1 hermaphrodites expressing CFP in their intestines were selected, and lines that transmitted the transgene were analyzed. At least three separate lines were analyzed for each injection.

Males expressing *unc-43* on a transgene were scored for spontaneous spicule protraction in the following manner: 6 L4 hermaphrodites for each transgenic line were placed on individual NGM plates containing *E. coli* OP50. F1 L4 males were selected,

allowed to mature overnight, and scored for spicule protraction. Late-stage L4 males containing UNC-43 driven by the heat-shock promoter were heat-shocked at 33°C for 0.5 hr, allowed to recover on NGM plates containing OP50 overnight and scored for spontaneous spicule protraction the next day.

To obtain worms with *egl-2* transgenic arrays, plasmids containing *egl-2* were injected into *unc-103(n1213);egl-2(rg4);him-5(e1490)* or *egl-2(rg4);slo-1(js379);him-5(e1490)* hermaphrodites. All injection mixtures contained 50 ng/μl of pBL66 and pUC18 was used to complete the mixtures to 200 ng/μl of DNA. Concentrations for the individual plasmids were as follows: 10 ng/μl of pBL122; 50 ng/μl of pBL109, pBL110, and pBL111; and 25 ng/μl of pBL108.

To determine the expression pattern of *acr-8*, pLR99 was inject into *pha-1(e2123);him-5(e1490)* hermaphrodites along with the pBX1 plasmid that contains wild-type *pha-1*. *pha-1(e2123)* is a temperature-sensitive allele that causes larval arrest at 20°C; therefore, only animals carrying the *pha-1(+)* gene on a transgenic array will survive [69]. Transgenic males containing pLR99 showed YFP expression in body wall muscles and a few unidentified neurons in the head, but no sex muscle expression.

Yeast two-hybrid assay

The yeast two-hybrid assay was performed as described in Matchmaker GAL4 Two-Hybrid System 3 & Libraries User Manual (PT3247-1, Clontech Laboratories, Inc., http://www.clontech.com). Briefly, the bait and prey plasmids were co-transfected into

Y187 yeast cells and plated on –Leu/-Trp minimal media plates to select for the presence of the plasmid. Detection of protein interaction was performed using the Galacton-Star reaction kit to test for the presence of β-galactosidase as described in the Yeast Protocols Handbook (PT3024-1, Clontech Laboratories, Inc.). Chemiluminesence produced by the β-galactosidase cleavage of the Galacton-Star reagent was read by the TopCount Microplate Scintillation Counter (Packard).

To detect the presence of the myc-tagged UNC-43 and ha-tagged EGL-2 proteins in yeast cells, the yeast containing the proteins was grown on –Leu/-Trp minimal media plates at 30°C for three days. The yeast cells were then scraped off the plate with 2 mL PBS buffer (phosphate buffered saline). The yeast cells were spun down and resuspended in 500 μl 1X laemmli buffer, after which they were boiled for 5 min to release the proteins. The boiled yeast cells were then loaded and run on an SDS-PAGE gel, transferred to a PVDF membrane, and probed for the presence of UNC-43 or EGL-2.

Protein purification

The EGL-2 c-terminus attached to MBP and UNC-43i attached to GST were purified in the following manner. All protein expression plasmids were transfected into *E. coli* BL21 cells. Cells were grown in Rich Media (10 g tryptone, 5 g yeast extract, 5 g NaCl, 2 g glucose, complete to 1 L) plus ampicillin at 37°C until they reached an OD_{600} of 0.5. Protein expression was induced by adding 500μl 1000X IPTG and the cells were

grown overnight at 25°C. The cells were harvested the next morning by spinning them down for 5 min at 5,000 rpm. Cells were re-suspended in 45 ml Column Buffer (20 ml 1.0 M Tris-HCl pH7.4, 11.7 g NaCl, 2.0 ml 0.5 M EDTA, complete to 1 L) and sonicated. Cells were spun down at 12,000 rpm for 10 min twice to obtain crude cell extract. We incubated crude cell extract with amylose or glutathione resin for 30 min with rotation at 4°C. We spun down the resin at 500 rpm for 2 min and removed the crude cell extract. Resin containing bound protein was washed 3X with 20 ml Column Buffer for 5 min rotating at 4°C, following by collecting resin at 500 rpm for 2 min. Protein-bound resin was transferred to two microcentrifuge tubes and 300 μl of Elution Buffer (Column Buffer plus either 10 mM maltose or 100 mM glutathione) was added to each tube. We incubated the mixture at 4°C rotating for 30 min, then spun down the resin and collected the elution, repeated 3X. EGL-2-MBP and UNC-43i-GST were also generated upon request from GenScript (http://www.genscript.com).

Protein interaction

I tested for direct *in vitro* interaction between EGL-2 and UNC-43i by determining if EGL-2-MBP could be co-purified with UNC-43i-GST and vice versa. The reaction was performed in 1X CaMKII Reaction Buffer (obtained by diluting 10X CaMKII Reaction Buffer, New England Biolabs, http://www.neb.com). 10X more UNC-43i was used than EGL-2. 1 mg of UNC-43i-GST was used per reaction, as was 144 μg EGL-2-MBP. Total reaction volume was 100 μl. To activated UNC-43i, the

following was added: 2 mM $CaCl_2$, 1.2 μM Calmodulin, and 200 μM ATP. The reaction mixture was incubated at 20°C overnight. To purify either UNC-43i-GST or EGL-2-MBP, 50 μl of the reaction was added to either 50 μl of glutathione resin (Sigma-Aldrich, http://www.sigmaaldrich.com) or 50 μl of amylose resin (New England Biolabs). The mixture was incubated at 4°C for 1 hr with rotation, after which the resin with the attached protein was collected by centrifugation. All centrifugation of the resin was performed at 500 rpm for 2 min at 4°C. The supernatant was removed and the resin was washed with 150 μl column buffer, incubated at 4°C with rotation for 5 min, and collected via centrifugation. This was repeated 2X. The flow-through was collected and concentrated to a final volume of 50 μl using Vivaspin 500 columns (Sartorius Stedim Biotech, http://www.sartorius-stedim.com). After the final wash, 50 μl of 100 mM glutathione (Sigma-Aldrich) or 50 μl of 10 mM maltose (Sigma-Aldrich) was added to the resin. The mixture was incubated at 4°C for 1 hr with rotation. The resin was collected and the supernatant removed. 5 μl of 10X Laemmli buffer was added to the supernatant and concentrated flow-through and the sample was boiled for 1 min. The sample was then run on a SDS-PAGE gel according to standard protocols [70] and transferred to PVDF membrane using a mini Trans-Blot electrophoretic transfer cell (Bio-Rad, http://www.bio-rad.com), allowing for the presence of the protein to be probed using standard Western blot protocols.

Western blot

Western blots were performed according to standard procedures. Anti-GST, 1:1000 (Abgent, http://www.abgent.com) and anti-MBP, 1:2000 (New England Biolabs), were used to detect the presence of UNC-43i-GST and EGL-2-MBP, respectively. Anti-HA, 1:1000 (Roche Applied Science, https://www.roche-applied-science.com/), Anti-myc, 1:1000 (Invitrogen), and Anti-KAP60 (courtesy of Dr. Kathy Ryan, Texas A&M University, College Station, TX) were used to detect the presence of EGL-2, UNC-43, and KAP60 in yeast cells, respectively. After removing excess primary antibodies, secondary antibodies Anti-Mouse and Anti-Rabbit IgG peroxidase conjugated (Thermo Scientific, http://www.thermo.com) were added at 1:5000. The Immun-star HRP chemiluminescent kit was used according to manufacturer instructions to visual the proteins (Bio-Rad). Film was developed using the Futura 2000K automatic x-ray film processor (Fisher Industries, Inc., Geneva, IL).

CHAPTER III

CAMKII/UNC-43 DOWN-REGULATES SEX MUSCLE EXCITABILITY IN A PATHWAY SEPARATE FROM ERG-LIKE K+ CHANNEL/UNC-103*

The *sy574* allele is in a gene that defines a pathway separate from ERG-like K+ channel *unc-103*

To discover how behaviors are coordinated at the molecular level, I studied the regulation of *C. elegans* male mating behavior. More specifically, I aimed to identify the regulatory molecules involved in preventing the inappropriate protraction of the male's sex organs, the spicules. In order for males to transfer sperm, the spicules need to stay inside the male tail until the hermaphrodite vulva has been breached. To identify molecules involved in regulating the timing of spicule protraction, mutations that cause males to display permanent protraction of their spicules were isolated. Males displaying this phenotype are unable to mate. The hermaphrodites of mutant lines kept for analysis appeared grossly normal while 40-80% of the males protracted their spicules [12]. The rate of spicule protraction was determined as follows. First, males were segregated from hermaphrodites at the L4 larval stage, before the copulatory structures have formed and

*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.

the stage immediately before they become adults. The males were kept on their standard food of *E. coli* OP50 overnight and scored as virgin adults the next day. A male scored as positive for protraction had at least part of one spicule protruding from the cloaca (See Chapter I, Figure 3). By identifying the molecular lesions that are responsible for in the mutant phenotype, I hope to discover how spicule protraction, and thereby behaviors more generally, are controlled at the molecular level.

The first mutation that results in spicule protraction identified was *sy557*. This mutation disrupts the function of the *ether-a-go-go* related gene (ERG) potassium channel, *unc-103* [12]. Uncoordinated (unc) animals display either slow movement or are paralyzed [4]. While *sy557* and other loss-of-function alleles of *unc-103* display wild-type movement, the *unc-103* gene was originally defined by a gain-of-function mutation that causes paralysis and egg-retention [71]. ERG-like K+ channels function in the vertebrate heart to assist in cell repolarization after a depolarization event has occurred [72-74]. Mutations in the human ERG K+ channels can lead to heart arrhythmias and sudden death [51,52]. The *C. elegans* ERG-like K+ channel *unc-103* functions to regulate the timing of sex-muscle contraction; disrupt its function, and males protract their spicules in the absence of mating cues.

A null mutation in *unc-103* (allele *n1213*, hereafter referred to as *unc-103(0)*) that deletes ~29 kilobases of the gene, including all transmembrane domains, results in 42% of males that protract their spicules (Table 2) [12,63]. The fact that 58% of males lacking *unc-103* still maintain their spicules inside their tails indicates the presence of one or more parallel pathways that can regulate the timing of sex-muscle contraction.

To identify molecules that compensate for the loss of *unc-103* function, I looked at another allele identified in the same mutant screen as *unc-103(sy557)*, *sy574*. Like *unc-103(sy557)*, *sy574* hermaphrodites do not display any gross abnormalities and males appear physically normal, but a portion display spicule protraction (56% of males; Table 2). I made a double mutant between *unc-103(0)* and *sy574* and found that disrupting function of these two genes results in nearly 100% of males protracting their spicules (Table 2) [49]. This indicates that the *sy574* lesion is in a gene with function separate from *unc-103*.

Table 2. Abnormal spicule protraction induced by mutant CaMKII/*unc-43* and ERG-like K+ channel/*unc-103* alleles

Genotype[a]	**% Protracted**	***p* Value**[b]
Wild-type	12 (106)	
ERG-like K+ channel/*unc-103(0)*	42 (91)	<0.005 to wt
CaMKII/*unc-43(sy574)*	56 (300)	<0.005 to wt
unc-103(0); unc-43(sy574)	97 (92)	<0.005 to wt
unc-103(sy557)	82 (55)	<0.005 to wt
unc-103(sy557); unc-43(sy574)	86 (65)	<0.005 to wt

[a]Strains contain *him-5(e1490)*
[b]Fisher's Exact Test

Interestingly, a double mutant between *sy574* and *unc-103(sy557)* results in 86% of males displaying the spicule protraction phenotype, significantly less than *unc-103(0);sy574* males (Table 2). The *unc-103(sy557)* lesion creates two amino acid changes: H165N in the linker region between transmembrane domains 2 and 3 and W244R in transmembrane domain 5 [12]. 82% of *unc-103(sy557)* males display spicule

protraction, compared to 42% for *unc-103(0)* males (Table 2). Thus, the *sy557* allele could result in an UNC-103 channel that interferes with parallel pathways present in *unc-103(0)* animals. Such parallel pathways prevent muscle seizures in 68% of males. In *unc-103(sy557);sy574* animals, both alleles could confer partial loss-of-function to their proteins, resulting in some males capable of suppressing premature spicule protraction. Further elaboration of *unc-103(sy557)*'s function is described in Appendix C.

The *sy574* lesion is in the CaMKII gene *unc-43*

To identify the location of the *sy574* lesion, I mapped the mutation to a 570-kb region on chromosome IV between cosmids R102 and K08F4 using single nucleotide polymorphism (SNP) mapping [60]. SNP mapping takes advantage of single nucleotide changes that exist between the *C. elegans* strain isolated by Sydney Brenner in England, N2, and a strain isolated in Hawaii, CB4856. *sy574* was crossed with CB4856 to generate lines that contain a mixture of N2 and CB4856 DNA. By determining a region where all the worms displaying *sy574*-induced spicule protraction are carrying N2 SNPs, I narrowed down the location of the *sy574* mutation. After determining that *sy574* was between cosmids R102 and K08F4, I identified candidate genes in the region. I then amplified these genes from wild-type genomic DNA by PCR and injected them into *sy574* hermaphrodites. I analyzed males carrying the wild-type genes on transgenic arrays to see if the genes could rescue *sy574*-induced spicule protraction. I found that

none of the genes reduced *sy574*-induced spicule protraction (Table 3). One gene, *unc-43*, covered a map area too large to amplify, so I analyzed the ability of *unc-43* alleles to rescue *sy574*-induced spicule protraction. I found that 4 out 5 *unc-43* alleles tested did not complement *sy574*, indicating that the *sy574* molecular lesion is in *unc-43* (Table 4) [49].

Table 3. Rescue of *sy574* using wild-type genes

Wild-type genes injected	**% Protracted**	***n***	***p* value**[a]
None	63	33	
gpa-7 + R09H10.6	74	31	0.4254
F13B12.3 + C43F9.6	79	28	0.2645
tax-6 + *cal-4*	97	32	0.0012

[a]Fisher's Exact Test

Table 4. Complementation tests between *sy574* and *unc-43* alleles

Genotype[a]	**% Protracted (*n*)**	***p* value**[b]
Wild-type	12 (106)	
unc-43(sy574)	56 (300)	
unc-43(sy574)/+	6 (79)	<0.005 to *unc-43(sy574)*
unc-43(n1186)	100 (58)	
unc-43(n1186)/+	6 (97)	<0.005 to *unc-43(n1186)*
unc-43(sy574)/*unc-43(n1186)*	60 (52)	
unc-43(n1179)	65 (34)	
unc-43(sy574)/*unc-43(n1179)*	54 (67)	
unc-43(sa200)	46 (31)	
unc-43(sy574)/*unc-43(sa200)*	33 (63)	
unc-43(e266)	98 (46)	
unc-43(sy574)/*unc-43(e266)*	42 (60)	
unc-43(e408)	11 (45)	<0.005 to *unc-43(sy574)*
unc-43(sy574)/*unc-43(e408)*	17 (72)	<0.005 to *unc-43(sy574)*

[a]Strains contain *him-5(e1490)*
[b]Fisher's Exact Test

The *unc-43* alleles tested all display spicule protraction defects on their own, except the complementing allele, *unc-43(e408)* (Table 4). In addition, these alleles, including the nonsense *unc-43(n1186)* allele that introduces an early stop codon, display other defects associated with disruption of *unc-43* function such as impaired movement and egg-laying behavior (Table 5). While disrupting the function of *unc-43* causes spontaneous spicule protraction, the males might still be able to mate before protraction occurs. To test the ability of males carrying *unc-43* mutations to mate, one virgin L4 male was placed with a paralyzed *unc-64(e246)* hermaphrodite. A male was scored as positive for mating if there was at least one moving progeny on the plate [4,75]. All *unc-43* alleles tested, except *sy574*, displayed reduced mating potency (Table 5). Since *unc-43(sy574)* males display abnormal spicule protraction but no other defects, it is likely that *sy574* is a weak loss-of-function allele. In addition, the spicule circuit appears to be more sensitive to perturbations in *unc-43* function.

Table 5. *unc-43* allele phenotypes

Allele[a]	Mutation	% Protracted (*n*)	Potency (*n*)	Unc	Spicule Insertion
Wild-type	none	12 (106)	86 (63)	-	yes
sy574	catalytic and association domains	56 (300)[b]	74 (61)	-	yes
sa200	unknown	46 (31)[b]	45 (41)[b]	-	yes
e408	catalytic domain	11 (45)	4 (68)[b]	+	no
e266	catalytic domain	98 (46)[b]	0 (38)[b]	+	ND
n1179	catalytic domain	65 (34)[b]	17 (36)[b]	+	ND
n1186	early nonsense	100 (58)[b]	0 (57)[b]	+	ND

[a]Strains contain *him-5(e1490)*
[b]*p* value <0.005 compared to wt

unc-43 encodes the only *C. elegans* copy of calcium/calmodulin-dependent protein kinase II (CaMKII), a serine/threonine kinase that phosphorylates a wide variety of substrates and is present in nearly all cell types [76]. CaMKII is primarily studied in vertebrate learning and memory and has unique structural properties [77,78]. CaMKII has three subunits: a kinase domain, an autoinhibitory domain, and a self-association domain (Figure 4A) [79]. The autoinhibitory domain keeps the kinase domain inactive in the absence of calcium and calmodulin. When calcium floods the cell and binds calmodulin, calmodulin then binds the autoinhibitory domain and the kinase domain is free to phosphorylate its substrates (Figure 4B-D). The self-association domain allows CaMKII to form 8 to 12 member complexes composed of two stacked rings (Figure 4E) [80,81]. Additionally, the autoinhibitory domain can be phosphorylated by other CaMKII molecules. When CaMKII is phosphorylated it is active in the absence of

calcium and calmodulin (Figure 4F). Members of these CaMKII complexes can then phosphorylate other members of the complex, helping to maintain enzyme activity in the absence of calcium. Thus, while a brief, infrequent calcium influx will activate CaMKII for a short amount of time, longer or frequent calcium signals will lead to constitutively active complexes of the enzyme. This is believed to be the basis for CaMKII function in learning and memory in brain cells [82,83]. The more an activity stimulates calcium influx, the more active CaMKII becomes. Active CaMKII then phosphorylates a range of substrates including transcription factors that lead to the molecular changes in neurons that help constitute the molecular basis for memory [84]. In this and most other instances where CaMKII has been studied, the enzyme activates molecules. In *C. elegans* male mating behavior, CaMKII is required to inhibit sex-muscle contraction until the appropriate mating cues are received.

To determine the nature of the molecular lesions in *unc-43(sy574)* animals, *unc-43* was sequenced from *sy574* genomic DNA. Two point mutations were found in *unc-43*: one changes glycine 170 to glutamate in the substrate recognition site of the catalytic region and the other changes alanine 465 to valine in the self-association domain (Figure 5) [85,86]. The *sy574* lesions could be affecting kinase function by interfering with the ability of *unc-43* to bind its substrates, the inability of *unc-43* to form complexes, or both. Additionally, I sequenced *unc-43(e408)* genomic DNA since the *e408* molecular lesion had not been previously identified. In addition, *e408* is the only allele that does not cause protraction and is able to complement *unc-43(sy574)* while disrupting the ability of males to sire progeny. *unc-43(e408)* contains one point mutation in the

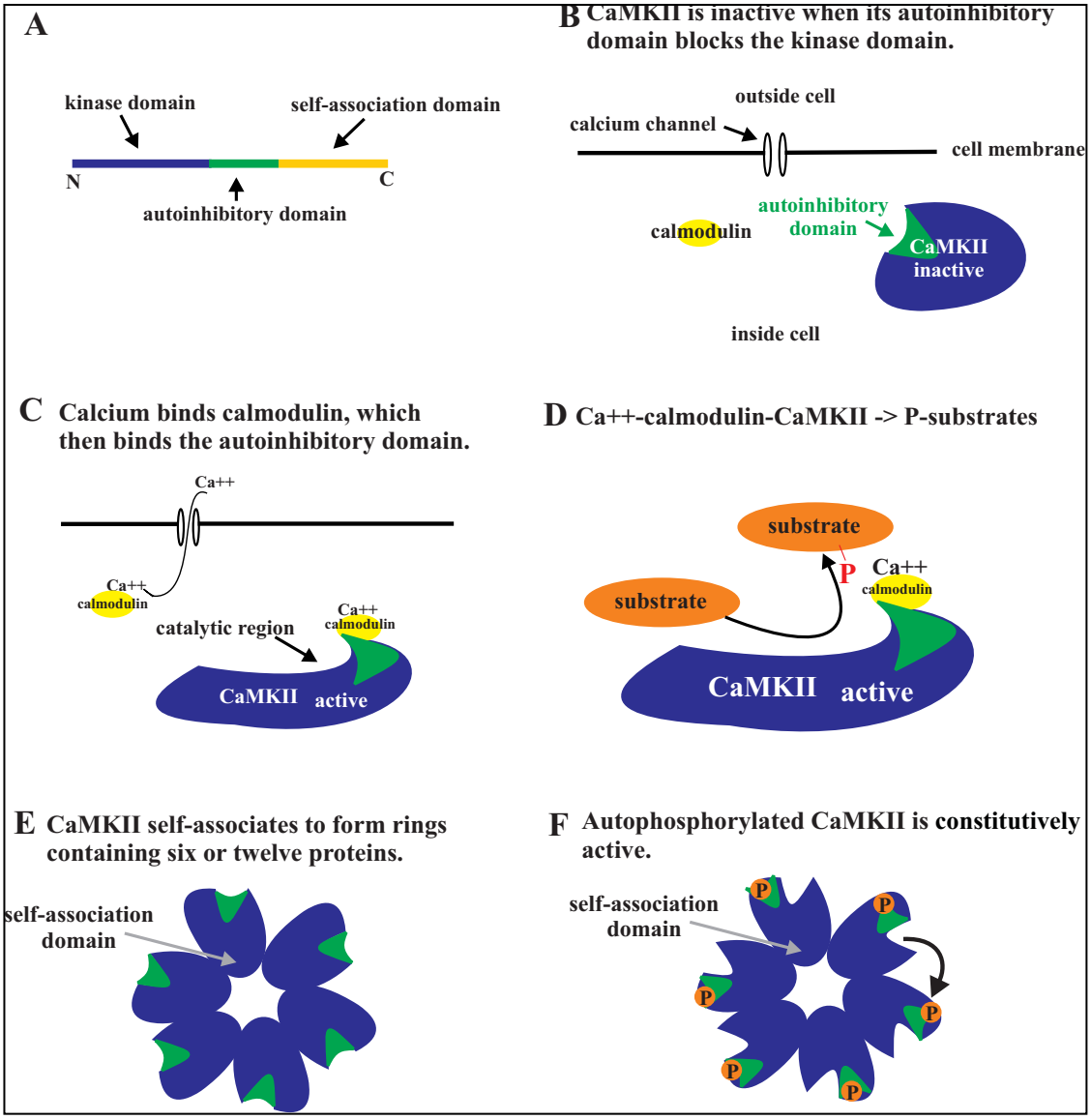

Figure 4. CaMKII structure and function. (A) Linear layout of domains from N- to C-terminals. The kinase domain is in purple, the autoinhibitory domain is in green, and the self-association domain is in yellow. (B) In the absence of calcium, the autoinhibitory domain (in green) sits in the kinase region, preventing the enzyme from phosphorylating substrates. (C) In the presence of calcium, calcium/calmodulin binds to the autoinhibitory domain and prevents it from interfering with the catalytic region of the kinase domain. (D) Activated CaMKII can then phosphorylate its substrates. (E) The self-association domain allows the kinase to form complexes of two stacked rings composed of six members each. The self-association domain is on the inside of the rings, while the kinase domain faces outward. (F) CaMKII can phosphorylate its own autoinhibitory domain, allowing the kinase to be active in the absence of calcium. This autophosphorylation can occur between members of the same 12-member complex.

substrate recognition domain of the catalytic region and changes a serine to leucine at amino acid 179 (Figure 5). This amino acid change is near the substrate recognition site of the catalytic region, suggesting the *e408* lesion interferes with the interaction between the kinase and its substrates.

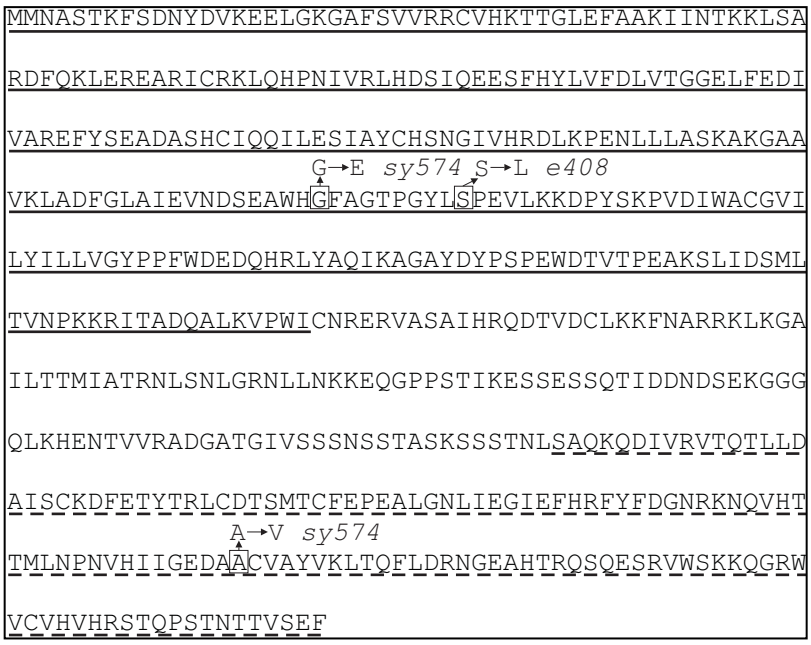

Figure 5. CaMKII/UNC-43 amino acid sequence with mutations. Wild-type amino acid sequence of UNC-43 isoform g (http://www.wormbase.org). The underlined region indicates the catalytic domain, no underline indicates the autoinhibitory domain, and the dashed underline indicates the self-association domain. The amino acids that are affected by the point mutations in *unc-43(sy574)* and *unc-43(e408)* are indicated by boxes. The amino acid change is listed next to the allele name [49].

CaMKII/UNC-43 is expressed in excitable cells in the male

Next I asked where CaMKII/*unc-43* is expressed in the male. Prior work using a rat anti-CaMKII antibody describes *unc-43* expression in hermaphrodite neurons, muscles, and intestines [76]. To determine where *unc-43* is expressed in the male, I amplified the full-length *unc-43* promoter by PCR. Because the 11-kb promoter proved too large to clone, I attached cyan fluorescent protein (CFP) to the promoter using PCR soeing (sequence overlap extension). I found that *unc-43* is expressed in excitable cells throughout the male (Figure 6A). In particular, *unc-43* is expressed in neurons and muscles in the male tail. The male's copulatory structures, located in the tail, consist of two spicules, each attached at their base by two retractor and protractor muscles [5]. In addition, the reorganized anal depressor muscle is also attached to the dorsal spicule protractors as a nonessential accessory muscle. The protractors are innervated by the SPC motor neuron which releases the acetylcholine (ACh) signal that causes muscle contraction [5,47]. Also innervating the system are the hook and post cloacal sensilla (p.c.s.) neurons that control spicule muscle function based on signals received from the environment [5,29]. *unc-43* is expressed in the SPC neuron as well as the p.c.s. neurons and the protractor and retractor muscles (Figure 6B). Therefore, *unc-43* could regulate spicule protraction in these cells.

In addition to the initial 5' UTR, the *unc-43* promoter region contains another 5' UTR 9-kb upstream of the start codon. To determine where the promoter upstream of the second 5' UTR drives expression, I cloned the 2.5-kb promoter region into a YFP-

containing vector and injected the 2.5-kb *unc-43* promoter:YFP into *pha-1(lf)* hermaphrodites along with wild-type *pha-1* to obtain transgenic worms. I then analyzed the 2.5-kb promoter expression pattern and found that *unc-43* is strongly expressed in the intestines of both sexes (Figure 6C-D). Additionally, *unc-43* is expressed from at least one unidentified neuron in the head that sends a process along the ventral side of the male (Figure 6D). In conclusion, *unc-43* is expressed in intestine, muscle, and neuronal cells in the male, similar to the expression pattern previously reported in the hermaphrodite [76].

CaMKII/UNC-43 and ERG-like K+ channel/UNC-103 suppress spicule protraction in muscles

Previous work done by Dr. L. Rene Garcia identified the expression pattern of *unc-103*. The *unc-103* gene contains six separate first exons and promoter regions (designated A-F). These promoters drive expression in most excitable cells in both the male and the hermaphrodite. One promoter in particular, $P_{unc\text{-}103E}$, expresses in the male sex muscles including the protractors and diagonal muscles used to position the male tail on the hermaphrodite as well as a few neurons in the head [63]. Thus, both *unc-103* and *unc-43* are expressed cells that control spicule protraction [49].

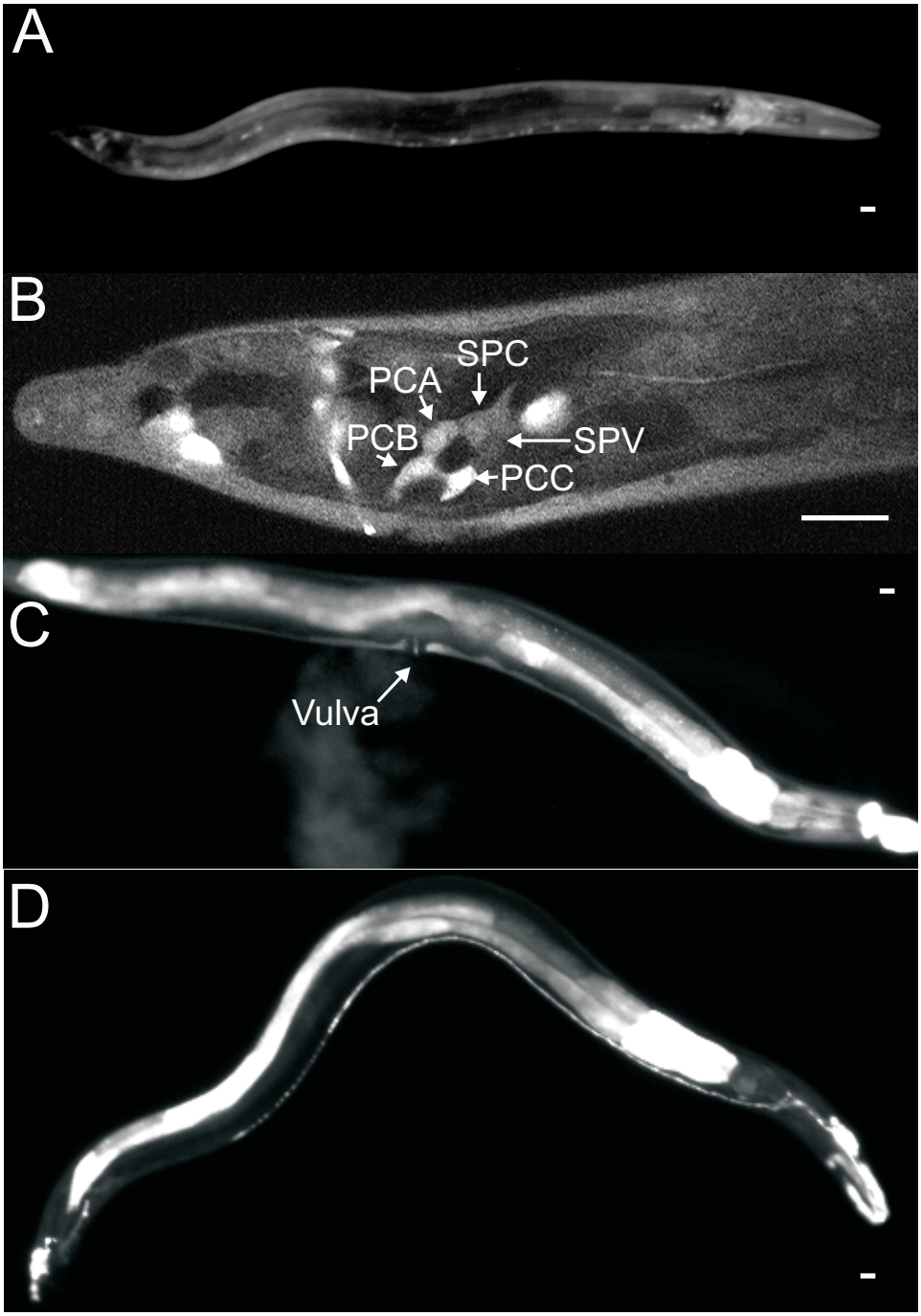

Figure 6. CaMKII/*unc-43* expression pattern. In all figures, anterior is to the right, dorsal is to the top. Scale bar = 10 μM. (A) Full-length *unc-43* promoter:CFP expression in a L4 (larval stage immediately preceding adulthood) male. (B) Full-length *unc-43* promoter:CFP expression in L4 male tail. Arrows indicate the neurons involved in mating. (C) 2.5-kb *unc-43* promoter:YFP expression in L4 hermaphrodite. The vulva is indicated. (D) 2.5-kb *unc-43* promoter:YFP expression in a L4 male [49].

To determine where *unc-103* and *unc-43* regulate protraction, wild-type genomic *unc-103* DNA and *unc-43* cDNA were expressed in various tissues. Dr. Garcia expressed *unc-103* in neurons using the P_{aex-3} promoter, in all muscles using the P_{lev-11} promoter, and in sex muscles using the $P_{unc-103E}$ promoter [63,65,66]. He found that *unc-103* expression in neurons was unable to rescue *unc-103(0)*-induced spicule protraction (Table 6). In contrast, *unc-103* expression in all muscles via the P_{lev-11} promoter reduced spicule protraction in *unc-103(0)* males from 39% to 3% (Table 6). To determine what subset of muscles *unc-103* functions in, *unc-103* was expressed in the sex muscles using the $P_{unc-103E}$ promoter. Dr. Garcia determined that sex muscle-specific rescue of *unc-103(0)* is sufficient to reduce sex-muscle excitability, as only 2% of *unc-103(0)* males expressing $P_{unc-103E}$:*unc-103(+)* protract their spicules (Table 6) [49].

Previous work determined that, in hermaphrodites, the *unc-103*E isoform expressed via the $P_{unc-103E}$ promoter is required to rescue an *unc-103(0)*-induced egg-laying defect [63]. Since the ability of *unc-103* genomic DNA to rescue an *unc-103(0)*-induced phenotype in the hermaphrodite is isoform specific, I asked if that was also the case in the male sex muscles. I expressed the *unc-103* isoform F in the sex muscles using the $P_{unc-103E}$ promoter and found that this construct reduced *unc-103(0)*-induced spicule protraction from 39% to 2%, similar to $P_{unc-103E}$:*unc-103E(+)* (Table 6) [49]. In conclusion, *unc-103* works in the sex muscles to regulate the timing of spicule protraction and this regulation is not isoform specific.

To determine where *unc-43* function is required to control spicule protraction, I expressed *unc-43* cDNA isoform g in different tissues. So far, seventeen different

isoforms of *unc-43* have been identified (http://www.wormbase.org) (Figure 7B). Most of these isoforms contain all three functional regions of CaMKII, and a lot of the variability between isoforms exists in the linker region (Figure 7A-B). However, some isoforms contain only the kinase or self-association domains, and there are two versions of the catalytic domain that differ in nine amino acids (Figure 7B-C). The *unc-43* cDNA isoform g was chosen because it contains all three CaMKII domains and has been used in other laboratories to rescue *unc-43* function [87,88] (Figure 7B). To determine if the *unc-43*g cDNA construct was able to rescue *unc-43(sy574)*-induced spicule protraction, I expressed it in all tissues using the heat shock promoter P_{hsp-16} [89,90]. Heat-shocked males expressing P_{hsp-16}:*unc-43*g showed a significant reduction in *unc-43(sy574)*-induced spicule protraction from control males (Table 6). However, the instance of protraction in *unc-43(sy574)* is not reduced to wild-type levels, suggesting that *unc-43* expression via the heat-shock promoter is either not turned on early enough or it is not expressed at a high enough level to fully reduce the instance of protraction. Having

Table 6. Tissue-specific rescue of mutant CaMKII/*unc-43* and ERG-like K+channel/*unc-103* –induced spicule protraction

genotype[a]	**Tissue expression**	**% Protracted (n)**	***p* value**[b]
CaMKII/*unc-43(sy574)*	not heat-shocked	56 (34)	
	heat-shocked	41 (34)	
*unc-43(sy574);rgEx*163 [P_{hsp-16}::*unc-43(+)*]	not heat-shocked	48 (23)	
	heat-shocked	24 (23)	p=0.03
*unc-43(sy574);rgEx*164 [P_{aex-3}::*unc-43(+)*]	pan neuronal	45 (60)	
*unc-43(sy574);rgEx*158 [P_{tnt-4}::*unc-43(+)*][c]	pharynx	57 (46)	
*unc-43(sy574);rgEx*161 [P_{lev-11}::*unc-43(+)*]	pan muscle	11 (98)	p<0.005
*unc-43(sy574);rgEx*120 [P_{lev-11}::*unc-43-self-asso. domain(+)*][c]	pan muscle	15 (61)	p<0.005
*unc-43(sy574);rgEx*159 [$P_{unc-103E}$::*unc-43(+)*]	spicule protractor muscles + few head neurons	59 (51)	
*unc-43(sy574);rgEx*174 [P_{acr-8}::*unc-43(+)*]	body-wall muscles + few head and ventral cord neurons	38 (37)	p=0.06
unc-103(0)[c]		39 (67)	
*unc-103(0);rgEx*74 [P_{aex-3}::*unc-103F(+)*][c]	pan neuronal	29 (53)	
*unc-103(0);rgEx*78 [$P_{unc-103F}$::*unc-103F(+)*][c]	*unc-103* specific neuronal expression	45 (40)	
*unc-103(0);rgEx*76 [P_{lev-11}::*unc-103F(+)*][c]	pan muscle	3 (30)	p<0.005
*unc-103(0);rgEx*79 [$P_{unc-103E}$::*unc-103F (+)*][c]	spicule protractor muscles + few head neurons	2 (44)	p<0.005
*unc-103(0);rgEx*81[$P_{unc-103E}$::*unc-103E (+)*][c]	spicule protractor muscles + few head neurons	2 (46)	p<0.005

[a]Strains contain *him-5(e1490)*
[b]Fisher's Exact Test
[c]Strain contains *pha-1(ts)*

determined that the *unc-43*g construct was functional, I proceeded with tissue-specific rescue of the *unc-43(sy574)* phenotype. The P_{aex-3} promoter was used to drive expression in all neuronal cells; the P_{tnt-4} promoter was used to drive expression in the pharynx, a muscular structure in the head of the worm used to grind food [28]; the P_{lev-11} promoter was used to drive expression in all muscle cells; the P_{acr-8} promoter was used to drive expression in body-wall muscles; and the $P_{unc-103E}$ promoter was used to drive expression in the sex muscles. I found that *unc-43*g expression in the neurons or pharynx was insufficient to reduce *unc-43(sy574)*-induced protraction (Table 6); in contrast, expression from the pan-muscle promoter P_{lev-11} reduced spicule protraction from 56% to 11%, indicating that *unc-43* functions in muscles to regulate the timing of sex-muscle contraction (Table 6). However, expressing *unc-43*g in specific subsets of muscles using the body-wall muscle promoter P_{acr-8} and the sex muscle promoter $P_{unc-103E}$ was insufficient to rescue *unc-43(sy574)*-induced protraction (Table 6) [49]. Thus, unlike *unc-103* that only functions in the sex muscles, *unc-43* is required in the body-wall and sex muscles to inhibit premature spicule protraction.

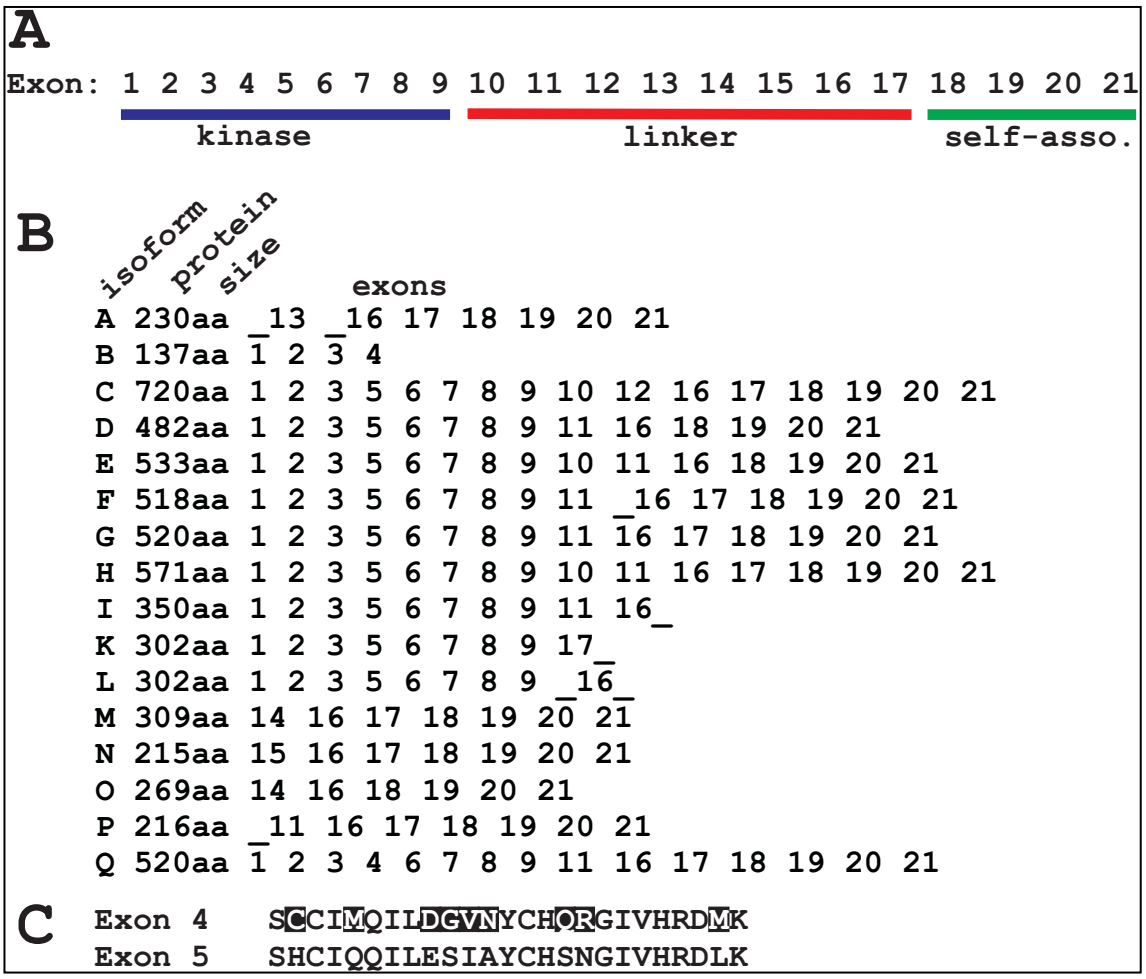

Figure 7. **CaMKII/*unc-43* isoforms.** (A) Exons in *unc-43* and what functional domains they define. Blue line under exons indicates they encode the kinase domain. Red line indicates the variable linker region. Green line indicates the self-association domain. (B) List of isoforms, their sizes, and the exons they contain. The exons are numbered according to the order they appear on the genomic DNA sequence. A "_" before or after an exon number indicates a truncated version of the exon is included in the isoform. "_11" lacks the first 18 base pairs, "_13" lacks the first 46 base pairs, "_16" lacks the first 6 base pairs, "16_" does not have last 84 base pairs, and "17_" does not have the last 96 base pairs. (C) Amino acid sequences encoded by exons 4 and 5. The amino acid differences between the two exons are indicated by black boxes.

The *unc-43(sy574)* allele contains two point mutations, one in the kinase domain (*sy574*A) and one in the self-association domain (*sy574*B) (Figure 5). To determine which mutation causes the spicule protraction phenotype, I expressed an *unc-43* construct lacking the self-association domain in the muscles. In addition to the full-length isoform *unc-43*g, *C. elegans* also contains an isoform lacking the CaMKII self-association domain, *unc-43*i (Figure 7B). I found that *unc-43*i expressed in muscles via the P_{lev-11} promoter reduces *unc-43(sy574)*-induced protraction from 56% to 15% (Table 6) [49]. In conclusion, the *sy574*A mutation, that changes glycine 170 to glutamate in the substrate recognition site of the catalytic domain, causes *unc-43(sy574)*-induced spicule protraction.

Chapter summary

In Chapter III, I demonstrated that a mutation in the *C. elegans* CaMKII gene, *unc-43*, causes permanent spicule protraction. *unc-43* is required to inhibit sex-muscle contraction until the male receives the appropriate mating cutes in a pathway separate from the ERG-like K+ channel encoded by *unc-103*. Interestingly, *unc-43*, required in body-wall and sex muscles, has a broader area of function to control spicule protraction than *unc-103*, which is only required in the male's sex muscles. When *unc-43* is expressed in all muscle cells via the P_{lev-11} promoter, *unc-43(sy574)*-induced spicule protraction is reduced. However, when it is expressed in either just the body-wall muscles via the P_{acr-8} promoter or just the sex muscles via the $P_{unc-103E}$ promoter, *unc-*

43(sy574)-induced protraction is unaffected. Thus, *unc-43* could be required in both the body-wall and sex muscles to control spicule protraction. Alternatively, because $P_{unc-103E}$ does not drive expression in the retractor muscles that are attached at the base of the spicules and help maintain their position inside the body, *unc-43* could be required in both the retractor and protractor muscles, unlike *unc-103* that is only required in the protractor muscles. In conclusion, the CaMKII gene *unc-43* inhibits sex-muscle excitability to prevent premature mating behaviors from occurring.

CHAPTER IV

CAMKII/UNC-43 DIRECTLY INTERACTS WITH EAG K+ CHANNEL/EGL-2 TO REGULATE SEX-MUSCLE EXCITABILITY*

An EAG K+ channel/*egl-2(gf)* mutation suppresses *unc-43(sy574)*-induced seizures

I wanted to identify direct molecular targets of UNC-43 in the *C. elegans* male sex muscles. CaMKII/UNC-43 is an enzyme that phosphorylates many different substrates to regulate a wide variety of biological processes, from learning and memory to heart muscle function [77,78,91,92]. By identifying direct down-stream targets of UNC-43 I can elucidate in greater detail how behaviors are controlled at the molecular level.

To address which molecules could be targets of CaMKII/UNC-43 in muscles to regulate the timing of spicule protraction, I tested if *unc-43* interacts with the *ether-a-go-go* (EAG) K+ channel *egl-2* [58]. I considered EAG K+ channels because they were shown to be a direct target of CaMKII in the *Drosophila* nervous system, as CaMKII phosphorylization up-regulates channel activity [93,94]. First, the location of *egl-2* was

*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.

determined to see if it is expressed in the same cells in the male as *unc-43*. A previous study identified *egl-2* expression in sensory neurons and sex muscles in the hermaphrodite [58]. *egl-2* is also expressed in sensory neurons in the male, as well as the sex muscles but not the neurons associated with spicule protraction (Figure 8) [49]. Therefore, *egl-2* could be a direct target of *unc-43* in the male sex muscles.

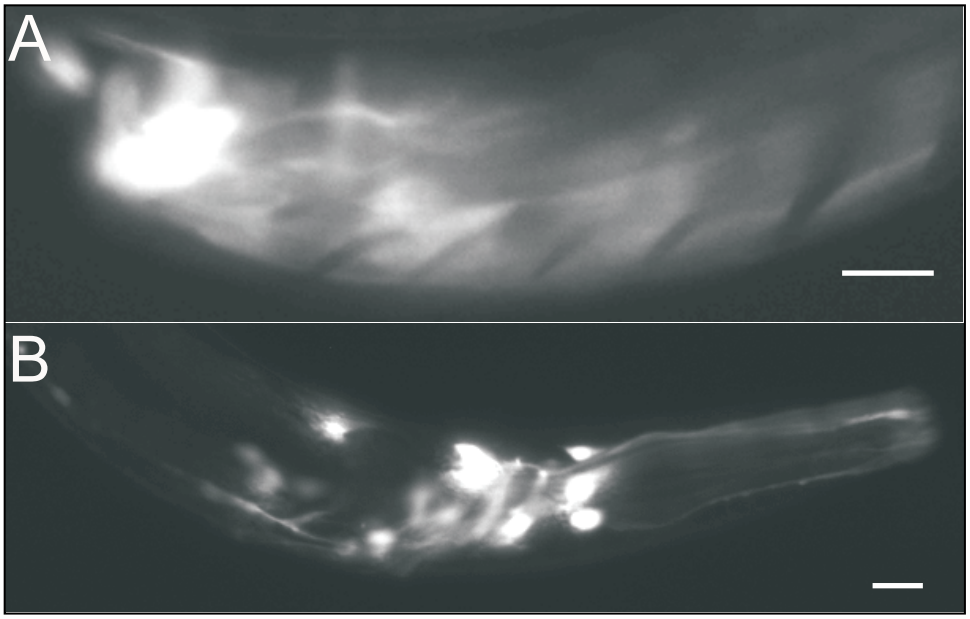

Figure 8. EAG K+ channel/*egl-2* expression pattern. In all figures, anterior is to the right, dorsal is to the top. Scale bar = 10 μM. (A) *egl-2* promoter:YFP expression in an adult male tail. (B) *egl-2* promoter:YFP expression in an adult male head [49].

Next I asked if there is an interaction between EAG K+ channel/*egl-2* and CaMKII/*unc-43*. I created a double mutant between an *egl-2* gain-of-function allele, *n693*, and *unc-43(sy574)* to determine if *egl-2* exhibited an effect on *unc-43(sy574)*-induced spicule protraction. I found that *egl-2(n693gf)*, an allele that causes

hermaphrodites to retain eggs but has no effect on spicule protraction in the males, is able to suppress *unc-43(sy574)*-induced spicule protraction. Since increasing the function of *egl-2* is able to compensate for the loss of function in *unc-43*, *unc-43* could activate *egl-2* to reduce spicule protraction in wild-type males (Table 7) [57]. Similarly, *egl-2(n693gf)* is able to partially suppress protraction caused by the nonsense allele *unc-43(n1186)* (Table 7). In contrast, *egl-2(n693gf)* has no effect on *unc-103(0)*-induced protraction, indicating that the *egl-2(gf)* allele does not have a general effect on sex muscle excitability (Table 7) [49]. However, over-expressing *egl-2(gf)* in the sex muscles using the $P_{unc\text{-}103E}$ promoter results in suppression of the *unc-103(0)* phenotype (Table 8). This suggests that while *egl-2* is not normally active under standard conditions in the sex muscles, over-expression of an active form of the K+ channel can reduce cell excitability. In conclusion, *egl-2* could be down stream of *unc-43* but does not function in the separate *unc-103*-mediated pathway controlling spicule protraction.

Table 7. Spicule protraction in EAG K+ channel/*egl-2* mutants		
Genotype[a]	**% Protracted (*n*)**	***p* Value**[b]
Wild-type	12 (106)	
CaMKII/unc-43(sy574)	38 (65)	<0.005 to wt
ERG-like K+ channel/*unc-103(0)*	43 (42)	<0.005 to wt
EAG K+ channel/*egl-2(n693gf)*	0 (30)	
unc-43(sy574); egl-2(n693gf)	4 (57)	<0.005 to *sy574*
unc-43(n1186)	100 (58)	<0.005 to wt
unc-43(n1186); egl-2(n693gf)	76 (50)	<0.005 to *n1186*
unc-103(0); egl-2(n693gf)	43 (94)	
egl-2(0)	11 (55)	
unc-43(sy574); egl-2(0)	15 (95)	<0.005 to *sy574*
egl-2(n693gf n904)	0 (30)	
unc-103(0); egl-2(n693gf n904)	14 (194)	<0.0001 to *unc-103(0)*
unc-43(sy574); egl-2(n693gf n904)	14 (49)	<0.006 to *sy574*

[a]Strains contain *him-5(e1490)*

[b]Fisher's Exact Test

EAG K+ channel/EGL-2 functions in the sex muscles to reduce seizures under food deprivation conditions

To determine the role of *egl-2* in spicule protraction, Dr. L. Rene Garcia generated a null mutation, *rg4*, that deletes a large portion of the *egl-2* gene (hereafter referred to as *egl-2(0)*). He found that removing the *egl-2* gene has no obvious effect on hermaphrodites or males, and does not cause spicule protraction (Table 7). Therefore, *egl-2* plays no identified role in regulating sex-muscle excitability under standard conditions.

To determine what role *egl-2* plays in the sex muscles, we looked at *egl-2*'s function under conditions of food deprivation. Previous work done by Todd Gruninger in the Garcia laboratory revealed that food deprivation is capable of reducing *unc-103(0)*-induced protraction from 36% to 9% (Table 8) [28]. He then asked if food-deprivation suppression of the *unc-103(0)* phenotype requires functional *egl-2*. *unc-103(0); egl-2(0)* double mutant males were analyzed for their ability to protract their spicules on plates containing no food. Whereas food deprivation reduces *unc-103(0)*-induced spicule protraction by 27%, it is unable to reduce protraction in *unc-103(0); egl-2(0)* males (Table 8). Therefore, *egl-2* is required under food deprivation conditions to reduce sex-muscle excitability.

I next asked where EAG K+ channel/*egl-2* functions to down-regulate muscle excitability under food deprivation conditions. I expressed wild-type *egl-2* cDNA in the sex muscles of *unc-103(0); egl-2(0)* males using the $P_{unc\text{-}103E}$ promoter [63]. I found that *egl-2* expressed in the sex muscles of double mutant males results in food deprivation-dependent suppression of *unc-103(0)*-induced spicule protraction (Table 8). Thus, *egl-2* functions in male sex muscles to reduce excitability when food is scarce.

Table 8. CaMKII/*unc-43* and EAG K+ channel/*egl-2* are required for food deprivation effects on spicule protraction

Genotype[a]	Food % Protracted (*n*)	No Food % Protracted (*n*)
Wild-type	12 (42)	0 (33)
unc-103(0)	43 (42)	14 (36)[b]
unc-103(0); egl-2(0)	58 (64)	40 (35)
unc-103(0); egl-2(0); rgEx173 [$P_{unc\text{-}103E}$*::egl-2(+)*]	49 (41)	9 (32)[b]
unc-43(sy574)	40 (234)	54 (173)[b]
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); rgEx255 [$P_{unc\text{-}103E}$*::egl-2(-end)*]	31 (36)	6 (36)[b]
unc-103(0); egl-2(0); rgEx258 [$P_{unc\text{-}103E}$*::egl-2(S567F)*]	9 (140)[c]	3 (34)
unc-103(0); egl-2(0); rgEx271 [$P_{unc\text{-}103E}$*::egl-2(gf)*]	6 (33)[c]	6 (33)
unc-103(0); egl-2(0); rgEx257 [$P_{unc\text{-}103E}$*::egl-2(S888F)*]	45 (31)	9 (34)[b]
unc-103(0); egl-2(0); rgEx268 [$P_{unc\text{-}103E}$*::egl-2(-PAS)*]	36 (36)	8 (49)[b]

[a]Strains contain *him-5(e1490)*

[b]*p* value < 0.05, Fisher's Exact Test compared to same genotype on food

[c]*p* value < 0.05, Fisher's Exact Test compared to *unc-103(0); egl-2(0); rgEx173* on food

Food deprivation-dependent reduction of sex muscle seizures requires CaMKII/UNC-43

Since both CaMKII/UNC-43 and EAG K+ channel/*egl-2* are activated under food-deprivation conditions and reduce sex muscle excitability, I asked if UNC-43 could

be involved in the activation of EGL-2 when food is limited. First, I determined if *unc-43* is required to down-regulate sex-muscle excitability under food deprivation conditions. To address this question, I generated double mutant combinations of *unc-103(0)* and two different *unc-43* alleles, *sy574* and *e408*. Both alleles were analyzed because they affect spicule protraction differently on food. Approximately half of *unc-43(sy574)* males display spontaneous protraction while *unc-43(e408)* males do not protract their spicules when in the presence of food (see Chapter III). In addition, *unc-43(e408)* is able to complement *unc-43(sy574)*-induced spicule protraction, indicating the two mutations disrupt different aspects of CaMKII function. Both alleles cause an amino acid change near the substrate recognition domain of the catalytic region, suggesting the *e408* and *sy574* mutations disrupt CaMKII's ability to recognize, and thereby phosphorylate, its substrates [49]. In both cases, combining the *unc-43* and *unc-103(0)* mutant alleles increases the instance of spicule protraction on food (Table 8). This is not surprising since genetic and pharmacologic data suggest the *unc-43(e408)* allele reduces male sex muscle function by up-regulating the UNC-103 and EGL-2 K+ channels (see Chapter V). EGL-2 activation does not suppress *unc-103(0)*-induced spicule protraction (Table 7), therefore *unc-43(e408)* does not have a K+ channel to activate when *unc-103* is removed, resulting in an increased instance of spicule protraction in *unc-103(0); unc-43(e408)* males. However, food deprivation is unable to reduce spicule protraction in *unc-103(0); unc-43(e408)* mutant males (Table 8). The inability of food deprivation to inhibit seizures in *unc-103(0); unc-43(e408)* males suggests *unc-43* inhibits sex muscle excitability when food is scarce. In contrast, food

deprivation reduces the instance of protraction seen in *unc-103(0); unc-43(sy574)* males (Table 8). This indicates that the UNC-43(*sy574*) kinase is likely able to inhibit the *unc-103(0)* phenotype, but not its own phenotype under food deprivation conditions. Interesting, food deprivation increases the instance of protraction in *unc-43(sy574)* single-mutant males (Table 8). This suggests that *unc-43* plays an increased role in reducing sex-muscle excitability when food is scarce. In conclusion, both the *sy574* and *e408* mutations can disrupt UNC-43's ability to transduce food deprivation signals (Figure 9).

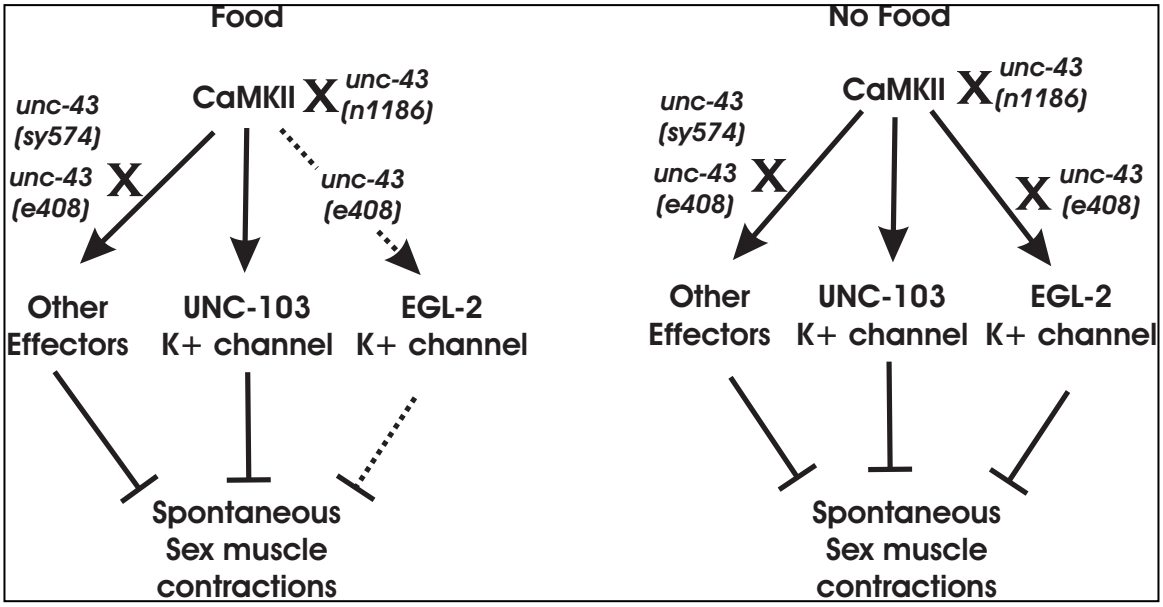

Figure 9. Genetic interactions between CaMKII, EAG, and ERG-like K+ channels in well-fed and food deprivation conditions. CaMKII acting upstream of K+ channels and other regulators of muscle contraction is represented by arrows, whereas bars indicate that the K+ channels and other effectors suppress spontaneous muscle seizures. An "X" next to an *unc-43* allele name denotes which specific genetic pathway is compromised by the mutant allele. The dashed arrow and bar lines indicate that the interaction between *unc-43* and *egl-2* is only seen in the *unc-43(e408)* mutant background [49].

Mutating potential CaMKII phosphorylation site S567 on EAG K+ channel/EGL-2 up-regulates the channel under well-fed conditions

My work has demonstrated that CaMKII/*unc-43* and EAG K+ channel/*egl-2* function in the sex muscles to reduce spicule protraction under food deprivation conditions. To determine if there is a direct interaction between *unc-43* and *egl-2*, I mutated potential CaMKII binding sites present in the *egl-2* c-terminus. CaMKII phosphorylates the consensus sequence of RXXS/T, and *egl-2* contains several potential sites (Figure 10) [58,95]. In addition, the PAS domain of *egl-2* was deleted. PAS domains are involved in sensing external stimuli and circadian rhythm function but have no known role in EAG K+ channels [96-98]. While the PAS domain contains no potential CaMKII binding sites, it could provide a docking site for proteins that CaMKII does phosphorylate, thus affecting EGL-2 function.

A cDNA encoding the mutated *egl-2* was expressed in the sex muscles of *unc-103(0); egl-2(0)* males using the $P_{unc\text{-}103E}$ promoter [63], and transgenic males were scored for the ability of food deprivation to inhibit *unc-103(0)*-induced spicule protraction. I found that mutating *egl-2* at sites S888 or S567, removing the final four potential CaMKII sites in the *egl-2* c-terminus, and removing the PAS domain all resulted in food-deprivation suppression of spicule protraction (Table 8). Thus, interfering with these sites does not inhibit the activation of EGL-2 in the absence of food.

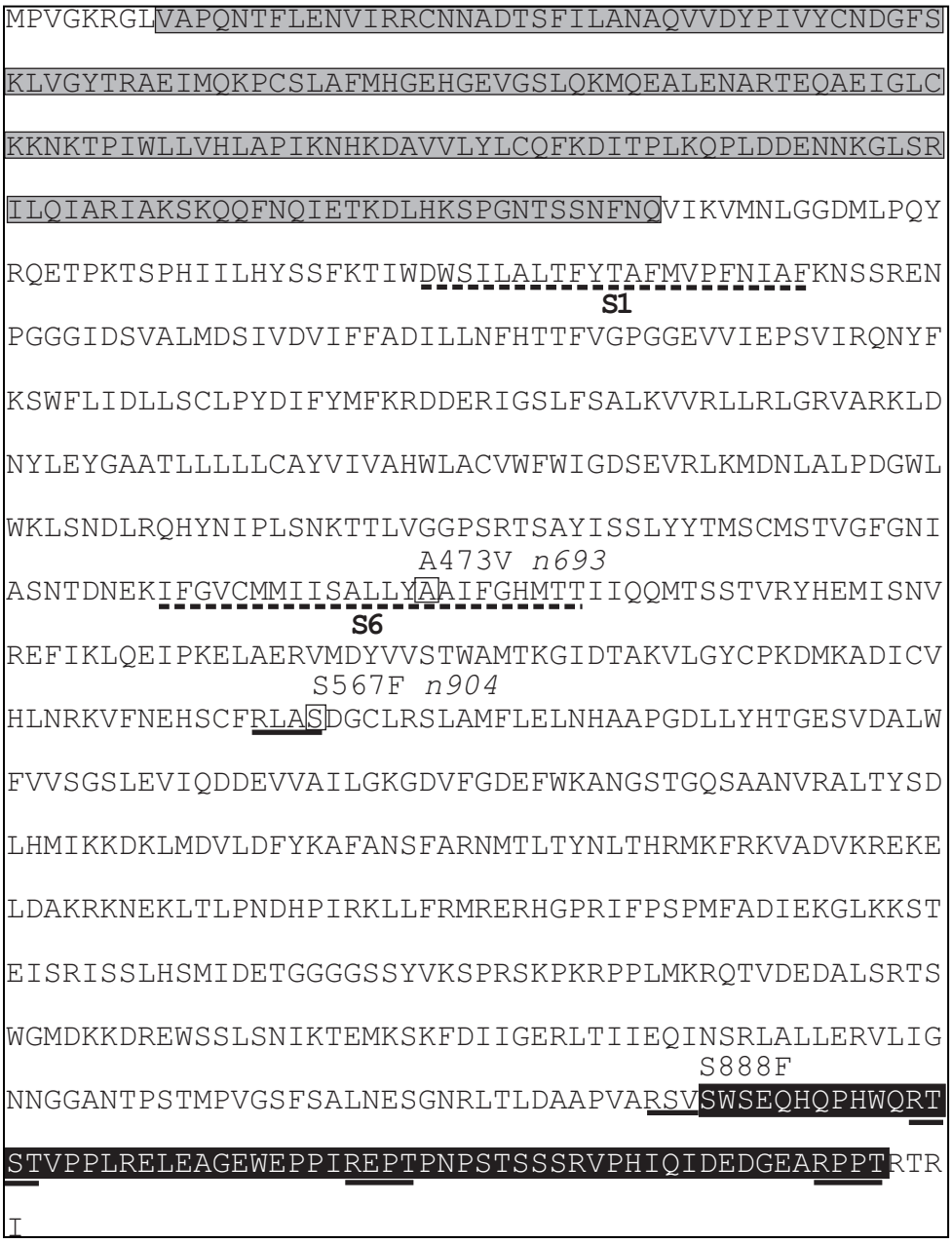

Figure 10. EAG K+ channel/*egl-2* amino acid sequence with mutations indicated. The PAS domain is highlight in gray. Dashed underline indicates transmembrane domains 1 and 6. Solid underline indicates potential CaMKII phosphorylation site. Black highlight indicates amino acids deleted in EGL-2(-del) mutant. Amino acids affected by mutations are boxed. Amino acid change as well as *egl-2* allele name is indicated above the amino acid affected.

However, under standard, well-fed conditions, mutating serine 567 to phenylalanine results in the suppression of *unc-103(0)*-induced protraction, as spicule protraction is reduced from 58% to 9% (Table 8). Interestingly, an allele of *egl-2* exists that is also able to inhibit spicule protraction on food. Unlike *egl-2(0)* and *egl-2(n693gf)*, *egl-2(n904)* reduces *unc-103(0)*-induced spicule protraction from 43% to 14% under well-fed conditions (Table 7). While the *egl-2(n904)* allele had previously been identified as a suppressor of the egg-laying defective phenotype caused by *egl-2(n693gf)*, the molecular lesion had not been identified [58]. Dr. Daisy Gualberto sequenced *egl-2* from *n904* worms and found serine 567 was changed to phenylalanine (Figure 10). Thus, males carrying the S567F mutation on the *n904* allele have a similar phenotype to expressing EGL-2 containing the S567F mutation in male sex muscles. *egl-2(n904)* and $P_{unc\text{-}103E}$:*egl-2(S567F)* both inhibit protraction induced by *unc-103(0)*. Thus, the S567F mutation results in active EGL-2 under standard, well-fed conditions, while wild-type EGL-2 is only active in the male sex muscles under food deprivation conditions (Figure 11).

Further support for the S567F mutation activating *egl-2* comes from the ability of *egl-2(n904)* to inhibit *unc-43(sy574)*-induced spicule protraction. The *unc-43(sy574)* allele contains a point mutation in the substrate recognition site of the kinase domain that could interfere with substrate binding and induces spicule protraction at a rate of 56% (Table 7) [49,85,86]. When the *unc-43(sy574)* allele is combined with the *egl-2(n904)* allele, male spicule protraction is reduced to 14% (Table 7). This supports the idea that EGL-2(*n904*) is active under well-fed conditions.

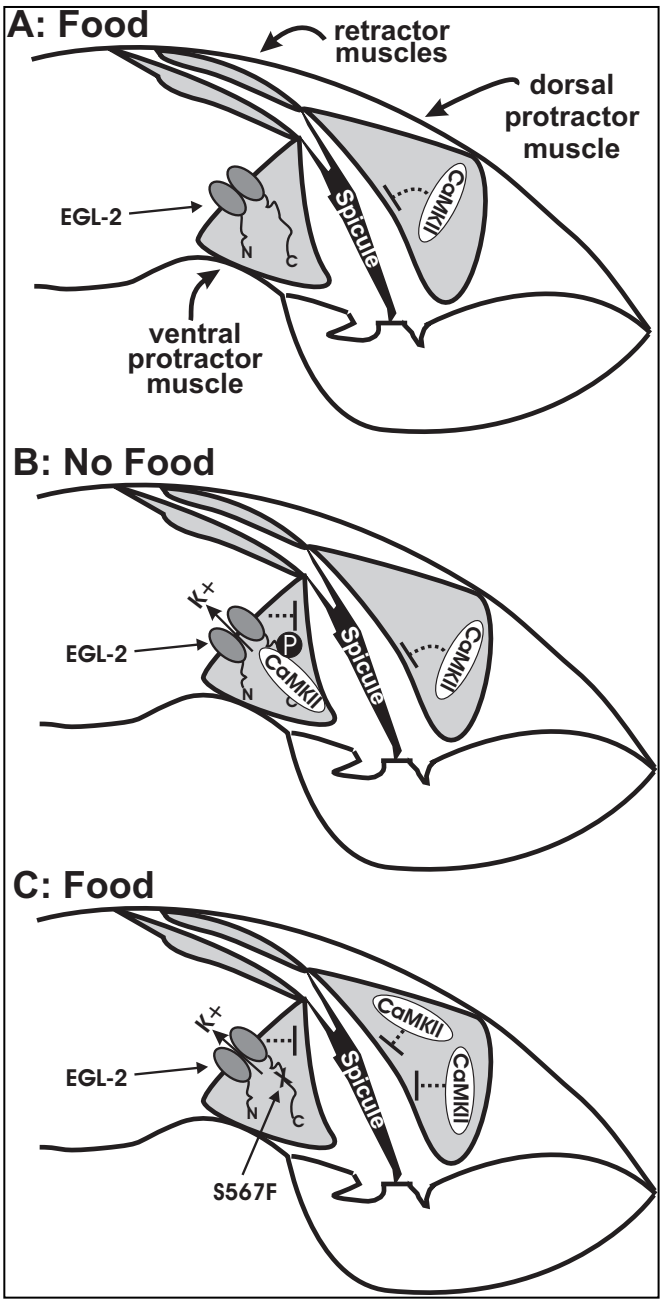

Figure 11. CaMKII activates EGL-2 under conditions of food deprivation to increase channel function. Diagram of the right half of the male tail. (A) When food is plentiful, CaMKII/UNC-43 inhibits sex muscle contraction and premature spicule protraction while EAG K+ channel/EGL-2 remains inactive. (B) When food is scarce, CaMKII phosphorylates EGL-2 at S567, up-regulating channel function to reduce sex muscle excitability. (C) Replacing serine 567 with phenylalanine results in up-regulated EGL-2 when food is plentiful. In addition to EGL-2 inhibiting premature spicule protraction, more CaMKII is present in the cytoplasm to reduce muscle seizures.

It has been shown in *Drosophila* that CaMKII not only phosphorylates EAG K+ channels, but also stays bound to the channel, indicating that EAG K+ channels can act as a "sink" for CaMKII. Thus, in males containing the *egl-2(n904)* mutation, not only could *egl-2* be active, but more CaMKII might be present in the cytoplasm since the *n904* allele could disrupt CaMKII binding. CaMKII could therefore be available in higher concentrations to phosphorylate other substrates, reducing sex-muscle excitability (Figure 11C). In support of this idea, combining *unc-43(sy574)* with a null allele of *egl-2* (*egl-2(0)*) results in reduced spicule protraction (Table 7). In *unc-43(sy574); egl-2(0)* males, more *unc-43* could be present in the cytoplasm, compensating for impaired function induced by the *sy574* mutation.

Whether CaMKII/UNC-43 binds and phosphorylates EAG K+ channel/EGL-2 at S567 to increase channel activity in *C. elegans* remains a question. Both EGL-2 and CaMKII/UNC-43 are required to reduce *unc-103(0)*-induced spicule protraction under food deprivation conditions, suggesting they are both active. Additionally, removing *egl-2* from a gain-of-function *unc-43* background increases the response of *unc-43(gf)* males to the acetylcholine agonist arecoline. When one-day-old virgin adult *unc-43(gf)* males are exposed to various concentrations of arecoline, 90% of them respond at 119 mM of the drug, compared to 579 μM required for 90% of wild-type males to respond [49]. The concentration increases to 9.7 mM for *unc-43(gf); egl-2(0)* males, indicating that UNC-43 is activating EGL-2 to reduce response to ARE. Thus, the above-mentioned lines of evidence indicate that UNC-43 is interacting with EGL-2 to increase channel activity (Figure 11B). However, mutating EGL-2 at the potential CaMKII

phosphorylation site S567 results in a channel able to suppress abnormal spicule protraction under well-fed conditions (Figure 11C). This indicates the S567F mutation increases the activity of EGL-2 under conditions when the wild-type K+ channel normally has little activity. The S567F mutation presumably disrupts the ability of CaMKII to phosphorylate the channel. However, phenylalanine contains a bulky side chain that could mimic the effects of phosphorylation, resulting in an active EGL-2 channel. Thus, the S567F mutation could activate EGL-2 in the presence of food because it is mimicking the phosphorylization by CaMKII that activates the channel under food deprivation conditions (Figure 11).

CaMKII/UNC-43 directly interacts with EAG K+ channel/EGL-2 in a Yeast Two-Hybrid assay and *in vitro*

To determine if the *C. elegans* proteins CaMKII/UNC-43 and EAG K+ channel/EGL-2 interact, I used a Yeast Two-Hybrid assay. An inactivated kinase form of UNC-43 and the EGL-2 c-terminal domain were attached to the GAL-4 binding and activation domains, respectively, and co-expressed in yeast cells. UNC-43 amino acid resides 1-270 were attached to the GAL-4 DNA binding domain (Figure 12A). The kinase was inactivated with an Asp 135 Asn mutation to prevent it from killing yeast cells [99]. The EGL-2 c-terminus consisting of amino acid residues 488-957, including the S567 potential CaMKII phosphorylation site, was attached to the GAL-4 activation domain (Figure 12A). Yeast cells containing both constructs were analyzed for their ability to produce β-galactosidase, the product of the lacZ gene. If UNC-43 directly

interacts with EGL-2, the binding and activation domains of GAL-4 will be brought close together, enabling transcription of lacZ and the production of β-galactosidase. I found there was a strong interaction between EGL-2 and UNC-43 in the Yeast Two-Hybrid assay, indicating there is a direct interaction between the two proteins (Figure 12B).

Since *in vivo* mutation of EGL-2 S567 interferes with normal channel function, I asked if mutating EGL-2 at S567 disrupts the interaction between EGL-2 and UNC-43. I placed a point mutation in the EGL-2-GAL-4 activation domain construct that changes S567 to phenylalanine and expressed EGL-2(S567F) in yeast cells along with UNC-43. I found almost no evidence of β-galactosidase production, indicating that the S567F mutation disrupts UNC-43's ability to bind EGL-2 (Figure 12B). To ensure that the reason I was seeing no β-galactosidase production was due to lack of protein interaction and not lack of protein expression in yeast cells, I probed for the presence of both EGL-2(S567F) and UNC-43. I found that both proteins were expressed (Figure 12C-D). In conclusion, the results of the Yeast Two-Hybrid assay indicate that UNC-43 is directly binding EGL-2 and S567, located in the EGL-2 c-terminus, is required for this interaction.

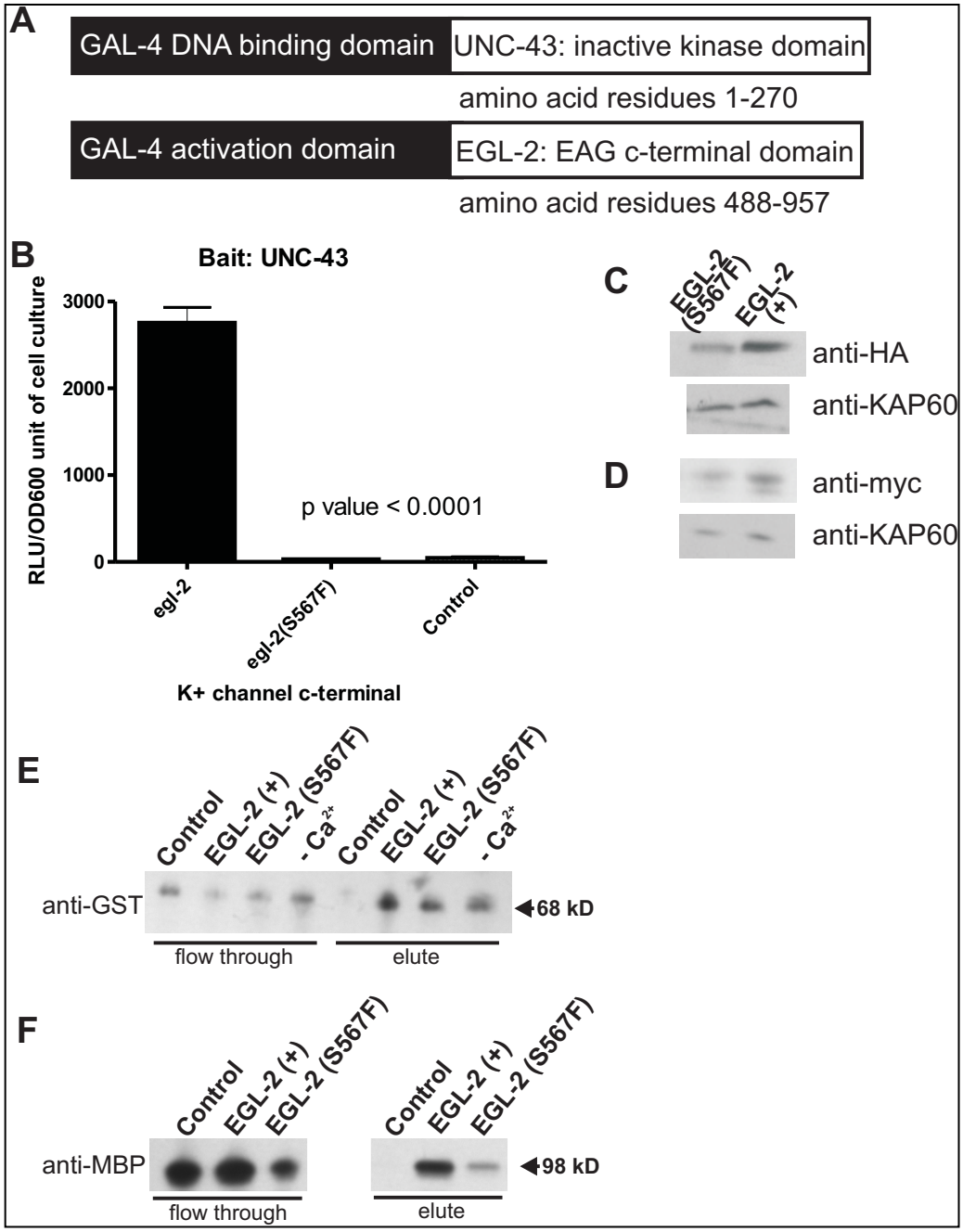

Figure 12. Direct interaction between CaMKII/UNC-43 and EAG K+ channel/EGL-2. (A) Diagram of UNC-43 and EGL-2 attached to the binding and activation domains of GAL-4. The amino acid residues used are indicated below the protein. (B) Protein interaction in a yeast two-hybrid assay. *x* axis is the c-terminus, *y* axis is the amount of real light units (RLU) given off per unit of cell culture. *p* value determined by Fisher's Exact Test. (C) Western blot testing for the presence of EGL-2-HA in yeast cells. (D) Western blot testing for the presence of UNC-43-myc in yeast cells. (E) Western blot testing for the presence of UNC-43-GST bound to EGL-2. (F) Western blot testing for the presence of EGL-2-MBP bound to UNC-43.

To confirm the direct interaction between UNC-43 and EGL-2, I looked at *in vitro* binding between the two proteins. I attached the UNC-43 kinase and regulatory domains to glutathione S-transferase (GST) and placed this construct in solution with the EGL-2 c-terminus, plus calcium and calmodulin, and asked if activated UNC-43 bound EGL-2. Using both amylose and glutathione resin, I separated UNC-43/EGL-2 complexes from unassociated UNC-43 (in the case of amylose resin) or EGL-2 (in the case of glutathione resin). I then used an antibody against maltose binding protein, to which EGL-2 was fused, to detect the presence of EGL-2 with eluted UNC-43, and an antibody against GST, to which UNC-43 is fused, to detect the presence of UNC-43. I found that EGL-2 is present in an elute of UNC-43; however, when EGL-2 S567 is mutated to F, EGL-2(S567F) is no longer present in an elute of UNC-43 (Figure 12F). UNC-43 is also present in an elute of EGL-2, and this interaction is abolished when the EGL-2 S567F mutation is present (Figure 12E). This confirms that *C. elegans* UNC-43 and EGL-2 interact, and this interaction is dependent upon EGL-2 S567.

Chapter summary

In this chapter I demonstrated that *C. elegans* CaMKII/UNC-43 and EAG K+ channel/EGL-2 directly interact and I provide evidence for the functional significance of this interaction. UNC-43 and EGL-2 are both expressed in male sex muscles and both are required to reduce muscle excitability under food deprivation conditions. UNC-43 interaction with EGL-2 is dependent upon S567, located in the K+ channel c-terminal

domain. Mutating serine 567 to phenylalanine appears to mimic phosphorylation, as the channel is then active under well-fed conditions and able to suppress muscle seizures. In wild-type males, EGL-2 displays little activity when food satiated, while when food is withheld EGL-2 could be directly activated by UNC-43 to inhibit seizures in the male sex muscles.

CHAPTER V

PATHWAYS ACTIVATED BY DIVERGENT FEEDING STATES TO REGULATE SPICULE PROTRACTION*

CaMKII/UNC-43 inhibits contraction by reducing sex muscle response to neurotransmitters

Further support for the role of CaMKII/*unc-43* in reducing muscle excitability comes from the ability of a loss-of-function allele in syntaxin/*unc-64* to inhibit *unc-43(sy574)*-induced protraction. Syntaxin is involved in docking neurotransmitter-filled vesicles with the cell membrane at the neuronal pre-synaptic terminal; reducing the function of syntaxin results in reduced neurotransmitter release [100,101]. I placed an *unc-64(lf)* allele with *unc-43(sy574)* and found that spicule protraction dropped from 56% to 17% in double-mutant males (Table 9). Since the site-of-action of *unc-43* in inhibiting spicule protraction is in the muscles, this indicates that *unc-43* is required in the muscles to reduce the response to neurotransmitter release.

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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.

Table 9. Abnormal spicule protraction reduced by mutations in Ca^{2+} channels		
Genotype[a]	**% Protracted (*n*)**	***p* Value**[b]
Wild-type	12 (106)	
syntaxin/*unc-64(lf)*	0 (41)	
CaMKII/*unc-43(sy574)*	56 (300)	<0.005 to wt
unc-64(lf); unc-43(sy574)	17 (81)	<0.0001 to *unc-43(sy574)*
L-VGCC/*egl-19(lf)*	2 (41)	
egl-19(lf); unc-43(sy574)	0 (48)	<0.005 to *unc-43(sy574)*
ERG/*unc-103(sy557)*	66 (454)	
unc-103(sy557); egl-19(lf)	5 (60)	<0.005 to *unc-103(sy557)*
RyR/*unc-68(0)*	15 (26)	
unc-43(sy574); unc-68(0)	46 (37)	
ERG-like K+ channel/*unc-103(0)*	42 (91)	<0.005 to wt
unc-103(0); unc-68(0)	8 (48)	<0.0001 to *unc-103(0)*

[a]Strains contain *him-5(e1490)*

[b]Fisher's Exact Test

CaMKII/UNC-43 reduces the effects of calcium influx from L-type voltage-gated Ca^{2+} channels under standard conditions

Previous work has established how the males' spicule muscles are activated during mating [47]. Protractor muscles attached at the base of the spicules are responsible for the prodding and insertion behaviors. The post cloaca sensilla PCB and PCC neurons sense the presence of the vulva and activate the protractor muscles by releasing the neurotransmitter acetylcholine (ACh). After the protractor muscles receive the activation signal, calcium is released from the sarcoplasmic reticulum via the Ryanodine receptor (RyR), UNC-68, resulting in the rapid prodding behavior required

for the spicules to breach the hermaphrodite vulva. Once the vulva is breached, the SPC motor neuron releases ACh, initiating calcium influx from the L-type voltage-gated Ca^{2+} channel (L-VGCC), EGL-19, that causes tonic contraction of the protractor muscles, resulting in spicule insertion into the vulva [47].

Since CaMKII/UNC-43 inhibits sex muscle contraction in the presence and absence of food, I asked if UNC-43 plays a role in reducing the effects of RyR/UNC-68 and L-VGCC/EGL-19 function. I placed an *egl-19* loss-of-function allele with *unc-43(sy574)* and found that *egl-19(lf)* abolishes *unc-43(sy574)*-induced spicule protraction under standard conditions (Table 9) [49]. Thus, when the worm is well-fed, *unc-43* is inhibiting calcium influx from *egl-19*. Similarly, *egl-19(lf)* is able to inhibit *unc-103(lf)*-induced spicule protraction (Table 9) [12]. Thus, *unc-43* and *unc-103* act redundantly to suppress the effects of calcium influx from *egl-19* in the absence of appropriate mating cues, preventing premature spicule protraction.

CaMKII/UNC-43 reduces the effects of calcium influx from Ryanodine receptors under conditions where food is scarce

I next made a double mutant between CaMKII/*unc-43(sy574)* and a null allele of RyR/*unc-68*. Unlike a loss-of-function in L-VGCC/*egl-19*, *unc-68(0)* is unable to suppress spicule protraction caused by the *unc-43(sy574)* allele (Table 9) [49]. Thus, *unc-43* does not reduce the effects of premature calcium influx from *unc-68* under standard conditions. In contrast, *unc-68(0)* inhibits spicule protraction caused by ERG-

like K+ channel/*unc-103(0)* (Table 9) [49], confirming that *unc-43* and *unc-103* are involved in separate pathways inhibiting sex-muscle seizures.

To test if CaMKII/*unc-43* plays a role in reducing the effects of RyR/*unc-68* calcium influx under food-deprivation conditions, I scored the spicule protraction of *unc-43(sy574); unc-68(0)* males raised on plates lacking the *E. coli* strain OP50. Food deprivation drops the instance of protraction seen in *unc-43(sy574); unc-68(0)* males from 49% to 12% (Table 10). This indicates that *unc-43* plays a role in reducing the amount of calcium released from *unc-68* in food-deprived males.

Table 10. Effects of RyR/*unc-68* mutants on spicule protraction under food deprivation conditions

Genotype[a]	**Food** **% Protracted (*n*)**	**No Food** **% Protracted (*n*)**
Wild-type	5 (38)	0 (26)
CaMKII/*unc-43(sy574)*	44 (66)	50 (44)
unc-43(sy574); unc-68(0)	46 (37)	12 (51)[b]

[a]Strains contain *him-5(e1490)*
[b]p value < 0.05, Fisher's Exact Test

To further test the role of CaMKII/*unc-43* in reducing the effects of calcium release from RyR/*unc-68* under conditions of food deprivation, I tested the response of *unc-43(sy574)* males to the acetylcholine agonist levamisole (LEV). LEV preferentially activates calcium release from UNC-68 [47]. Therefore, if *unc-43* reduces UNC-68

function when food is scarce, food-deprived *unc-43(sy574)* males should show an increased response to LEV since *unc-43*'s ability to reduce the amount of calcium released from *unc-68* is impaired. Food-deprived one-day-old virgin *unc-43(sy574)* males that did not display spontaneous spicule protraction were placed in various concentrations of LEV, observed for spicule protraction, and compared to similarly treated wild-type males. Food-deprived *unc-43(sy574)* males showed a significant increase in their response to 1 μM LEV (Figure 13B), supporting the idea that *unc-43* reduces the effects of *unc-68*-induced calcium influx under conditions of food deprivation.

Interestingly, *unc-43(sy574)* also displays hypersensitivity to LEV when males are food satiated (Figure 13A). This suggests that UNC-43 may play a role in reducing calcium influx from UNC-68, despite the fact that a null mutation in *unc-68* does not reduce *unc-43(sy574)*-induced protraction. Unidentified isoforms of *unc-68* could exist that are still present in *unc-68(0)* males. Alternatively, the *unc-43(sy574)* allele could cause a general increase in sex-muscle excitability, resulting in males that are more sensitive to ACh agonists. The hypersensitivity displayed by *unc-43(sy574)* males to LEV suggests a role for *unc-43* in regulating *unc-68*.

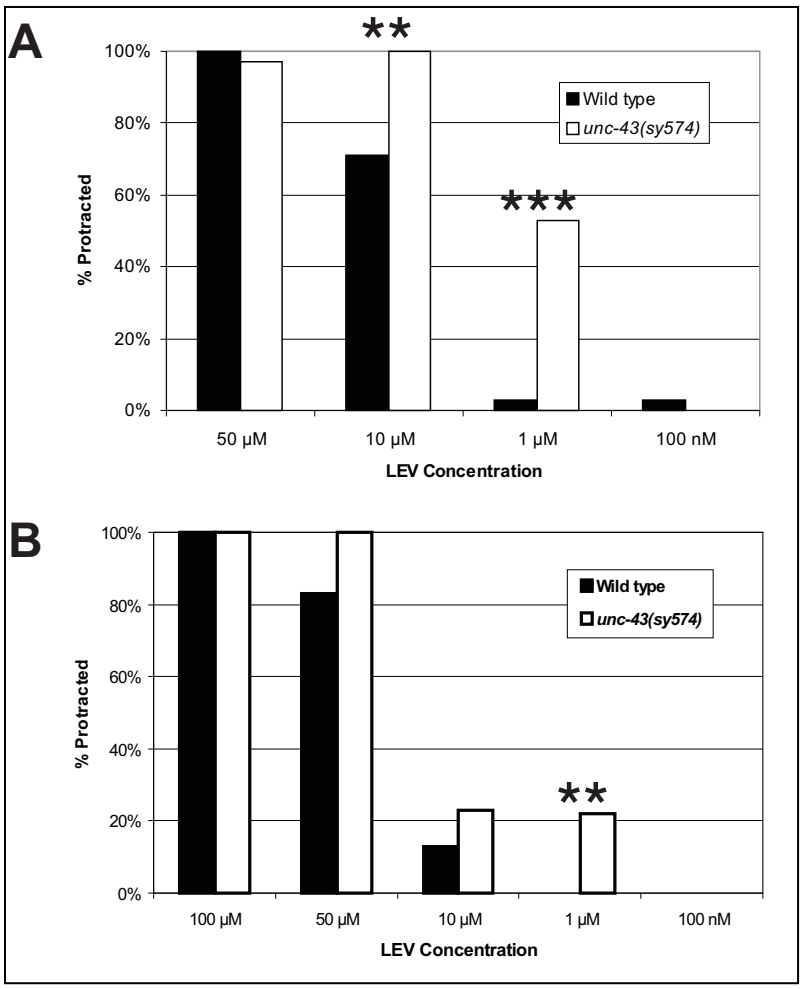

Figure 13. Levamisole pharmacology of well-fed and food-deprived males. *x*-axis is the drug concentration and the *y*-axis indicates the percent of males that protract their spicules. ** *p* value <0.005, *** *p* value < 0.0005, Fisher's Exact Test. (A) Well-fed male response to LEV. $n > 30$ for each bar. (B) Food-deprived male response to LEV. $n = 30$ for each bar, except 1 µM $n = 60$ for both genotypes.

ERG-like K+ channel/UNC-103 and EAG K+ channel/EGL-2 work downstream of CaMKII/UNC-43 to reduce sex muscle excitability

Under standard conditions, CaMKII/UNC-43 inhibits premature sex-muscle contraction by reducing the effects of calcium influx from L-VGCC/EGL-19. This calcium influx is preferentially activated by the ACh agonist arecoline (ARE) [47]. To dissect UNC-43's role in reducing calcium influx from EGL-19, I looked at the response of mutant *unc-43* alleles to ARE. One-day-old virgin males that maintained normal spicule position were placed in various concentrations of ARE and observed for protraction. 90% of *unc-43(sy574)* males displayed protraction at a concentration of 268 μM (EC_{90}), similar to the wild-type EC_{90} of 579 μM (Figure 14A,D). Since wild-type and *unc-43(sy574)* males have similar dose-response curves to ARE, this confirms that the *unc-43(sy574)* allele has no known phenotypes outside of spicule protraction, unlike other *unc-43* alleles. Two other *unc-43* alleles tested, the dominant gain-of-function allele *unc-43(n498gf)* and the loss-of-function allele *unc-43(e408)*, displayed greater than 10-fold reduction in response to ARE (Figure 14A,D). This indicates that *unc-43(n498gf)* and *unc-43(e408)* are over-active in suppressing sex-muscle excitability. In addition, *unc-43(408)*, while nominally a loss-of-function allele, displays gain-of-function attributes in the spicule protraction circuit; *unc-43(e408)* is the only allele that does not cause spicule protraction and the allele complements *unc-43(sy574)* (See Chapter III) [49]. This unique allele changes a serine to leucine at amino acid 179 in the

catalytic region and might be affecting *unc-43*'s ability to bind and phosphorylate substrates.

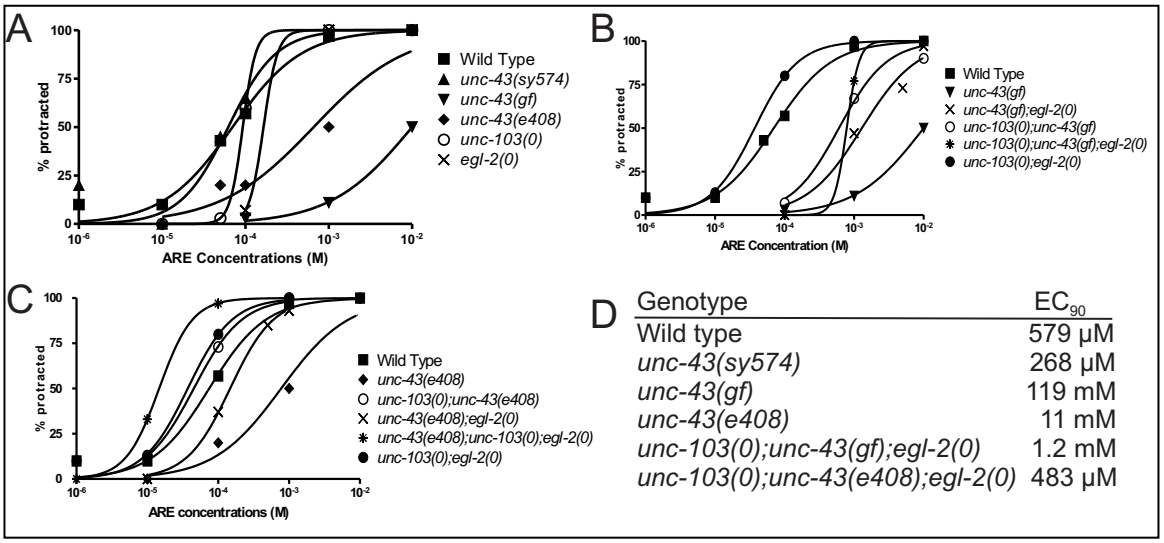

Figure 14. Arecoline pharmacology of CaMKII/*unc-43* mutants. (A-C) Graphs display male muscle sensitivity to ARE. The concentrations of ARE are listed on the *x*-axis and the percentage of males that protract their spicules in response to ARE is indicated on the *y*-axis. For each data point, $n = 30$ males. (D) EC_{90}: Concentrations at which 90% of males protract their spicules in response to ARE [49].

I next asked which proteins mediate ARE resistance induced by *unc-43(n498gf)* and *unc-43(e408)*. I looked at the interaction between *unc-43* and two K+ channels, the ERG-like K+ channel/*unc-103* and the EAG K+ channel/*egl-2*. *unc-103* was established as being downstream of *unc-43* in the defecation circuit, but so far my experimental results place *unc-103* and *unc-43* in separate pathways that suppress seizures in the spicule protraction circuit [49,76]. I have already established that *egl-2* is activated by *unc-43* under conditions of food deprivation; addressing the role of *egl-2* in *unc-*

43(n498gf) and *unc-43(e408)*-induced ARE resistance will allow me to determine if the K+ channel can be activated by these abnormally functioning kinases under well-fed conditions. I placed the null mutations of both K+ channels in *unc-43(n498gf)* and *unc-43(e408)* backgrounds and determined the double mutant's sensitivity to ARE. I found that removing either *unc-103* or *egl-2* from the *unc-43(e408)* background completely restores males' sensitivity to ARE while removing *unc-103* or *egl-2* from the *unc-43(n498gf)* background partially restores males' ARE sensitivity (Figure 14B-C). Thus, *unc-103* and *egl-2* are being activated by *unc-43* to reduce sex muscle excitability in the spicule protraction circuit. In addition, *unc-43(e408)* is a weaker gain-of-function allele in the spicule protraction circuit than *unc-43(n498gf)*.

While removing either *unc-103* or *egl-2* is sufficient to restore *unc-43(e408)* male's spicule muscles sensitivity to ARE to wild-type levels, the same is not true for *unc-43(n498gf)* males. I asked if removing both *unc-103* and *egl-2* from an *unc-43(n498)* background is sufficient to restore males' response to ARE. I made an *unc-103(0); unc-43(n498gf); egl-2(0)* triple mutant and found that while this increase sex muscle response greater than deleting either K+ channel alone, it did not restore sensitivity to wild-type levels (Figure 14C-D). Thus, *unc-43(n498gf)* is activating an unidentified protein(s) to suppress male sex-muscle response to ARE in addition to *unc-103* and *egl-2*.

EAG K+ channel/EGL-2 is activated by food deprivation to inhibit L-type voltage-gated Ca^{2+} channels

To confirm EAG K+ channel/EGL-2's role in reducing sex-muscle excitability by reducing the effects of calcium influx from L-VGCC/EGL-19, I looked at the response of *egl-2(gf)* mutant males to the EGL-19-activating ACh agonist ARE. Mutant males were placed in various concentrations of the drug and scored for spicule protraction. I found that *egl-2(gf)* showed reduced sex-muscle sensitivity to ARE at lower concentrations of the drug, but retained its sensitivity to higher concentrations (Figure 15B). In contrast, *egl-2(gf)* sensitivity was unchanged in response to another ACh agonist, levamisole, that activates calcium influx via RyR/UNC-68 (Figure 15A). This confirms that EGL-2 is involved in reducing the effects of L-VGCCs and not RyRs.

While EGL-2 inhibits L-VGCCs under food deprivation conditions, the EAG K+ channel does not appear to play any role in reducing sex-muscle excitability on food, as neither *egl-2(0)* nor *egl-2(gf)* males display mating defects. To test this idea, I asked if food-deprived *egl-2(gf)* males retain their resistance to the ACh agonist ARE after a period of re-feeding. Previous work established that male sex-muscle excitability is reduced when food is withheld, and this reduced excitability includes a decreased sensitivity to ARE [28,75]. *egl-2(gf)* males appear mostly normal on food; this could be due to the fact that *egl-2* is not active on food. Up-regulating EGL-2 activity in the male sex muscles off of food to inhibit sex-muscle excitability should up-regulate the previously dormant *egl-2(gf)*. Since it is a gain-of-function, it should remain active even

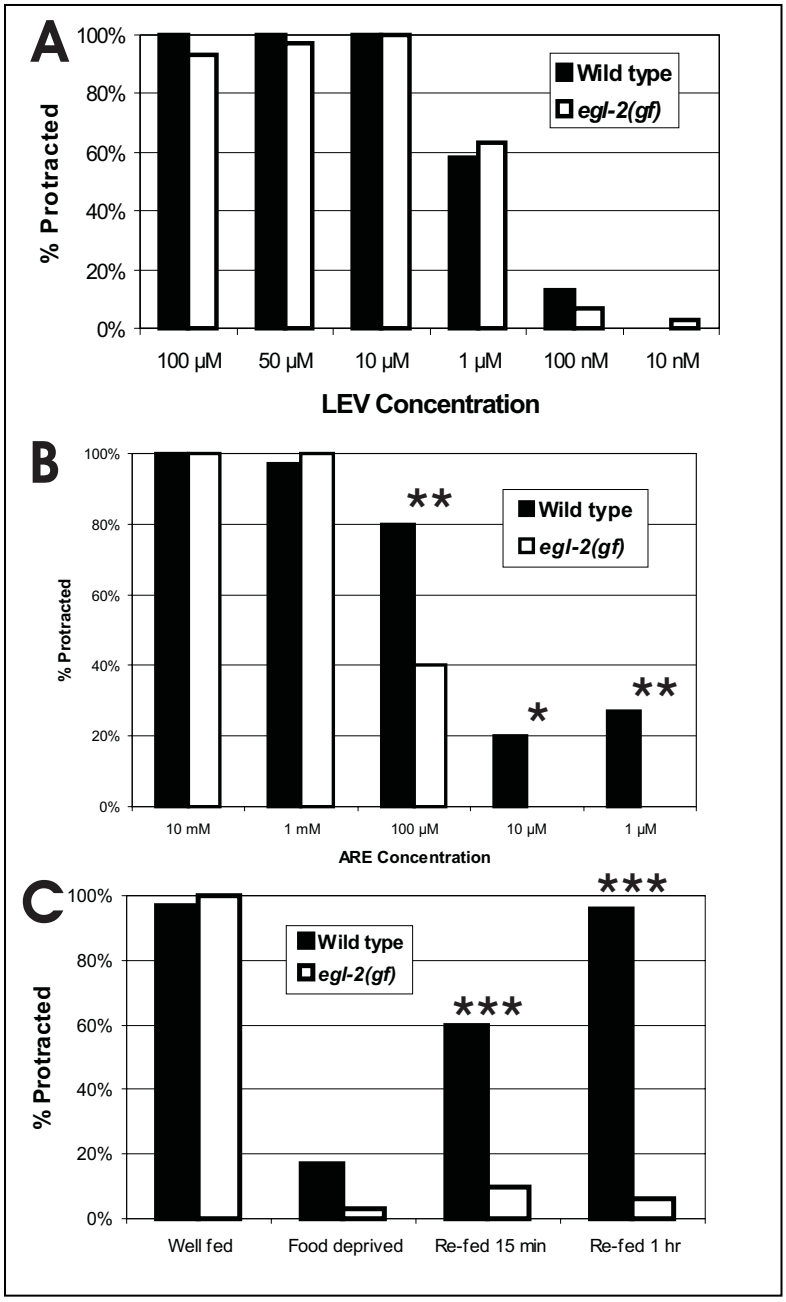

Figure 15. EAG K+ channel/*egl-2(gf)* sensitivity to ACh agonists. (A-B) Drug concentration is on the *x*-axis, and the percent of males that protracted their spicules in response to LEV is on the *y*-axis. $n = 30$ for all points tested. (A) *egl-2(gf)* sensitivity to LEV. (B) *egl-2(gf)* sensitivity to ARE. (C) Response to 1 mM ARE. Feeding status is indicated on the *x*-axis. The instance of spicule protraction in response to 1 mM ARE is indicated on the *y*-axis. For all graphs, * $p<0.05$, ** $p<0.005$, *** $p<0.0005$, and all strains contain *him-5(e1490)*.

when the male is returned to food. To this hypothesis, I food-deprived wild-type and *egl-2(gf)* males and re-fed them for a period of 15 min or 1 hr, and then tested sex-muscle response by looking for spicule protraction when the males were placed in 1 mM ARE. 1 mM ARE was used because *egl-2(gf)* males respond normally to this concentration of ARE when food satiated (Figure 15B). I found that wild-type males regain ARE sensitivity after 1 hr re-feeding, while *egl-2(gf)* males maintain their resistance to the drug (Figure 15C). Thus, *egl-2* is being activated by food deprivation and in wild-type males this activation is reversed upon the return to a food source.

CaMKII/UNC-43 requires BK K+ channel/ SLO-1 to regulate sex-muscle excitability

To identify other targets of CaMKII/*unc-43* in an *unc-43(n498gf)* background, thereby helping to identify relevant *unc-43* effectors in the male sex muscle circuit, I looked at the role of the BK K+ channel/*slo-1* in regulating spicule protraction. *slo-1* is expressed in neurons and muscles and was previously identified in a screen for suppressors of *unc-64*/syntaxin-mediated resistance to volatile general anesthetics, as was *unc-43* [59,102]. In addition, *slo-1* has been shown to be downstream of *unc-43* at the *C. elegans* presynaptic nerve terminal, as *unc-43* is involved in activating *slo-1* to inhibit neurotransmitter release [103]; a similar mechanism could function in the sex muscles. First, I looked at the ability of a *slo-1(lf)* nonsense allele to cause premature spicule protraction and found that *slo-1(lf)* causes 29% of males to protract their spicules

(n = 53, p value < 0.0001, Fisher's Exact Test compared to wild type). Therefore, *slo-1* inhibits inappropriate male sex muscle contraction under standard conditions.

To determine if *slo-1* functions downstream of *unc-43* to regulate spicule protraction, I made a double mutant between *unc-43(n498gf)* and *slo-1(lf)*. I then tested the ability of ARE to induce sex muscle contraction in these males. I found that the *slo-1(lf)* mutation was able to increase *unc-43(n498gf)*'s response to ARE (Figure 16), indicating that *unc-43* relies on *slo-1* to suppress muscle excitability in the spicule protraction circuit.

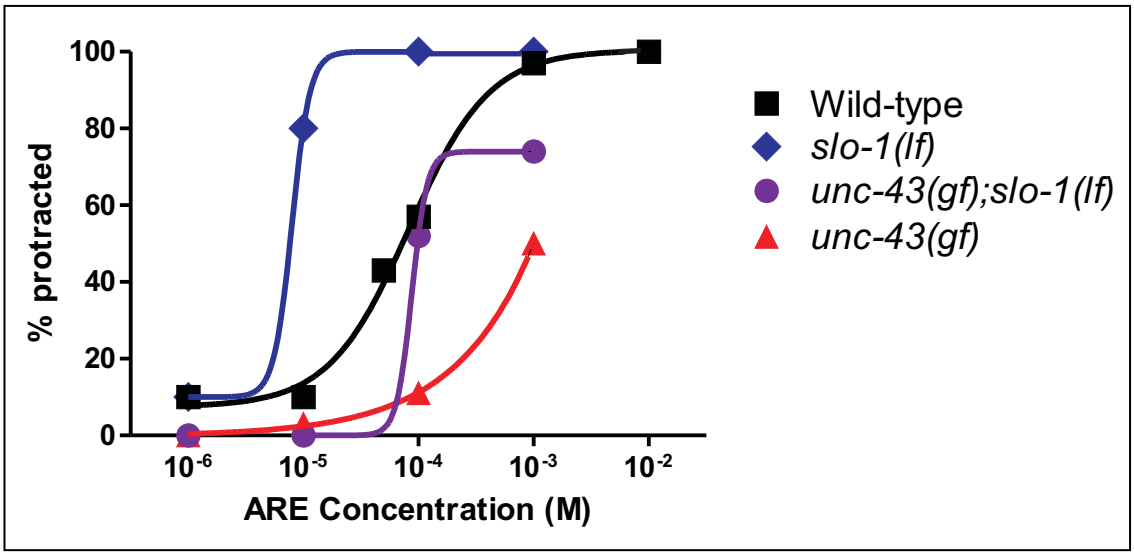

Figure 16. Arecoline pharmacology of BK K+ channel/*slo-1* mutant. ARE concentration on the *x*-axis, percentage of males that protracted their spicules in response to ARE on the *y*-axis. n = 30 for each data point.

Under food deprivation conditions, BK K+ channel/SLO-1 defines a pathway separate from CaMKII/UNC-43 and EAG K+ channel/EGL-2

Since *unc-43* is required to suppress spicule protraction not only under standard conditions, but also when food is scarce, I asked if *slo-1* plays a role in down-regulating sex-muscle excitability under food deprivation conditions. After starving virgin males overnight as they matured into adults, I found that food deprivation did not affect the instance of spicule protraction (Table 11). Thus, *slo-1* does play a role in reducing sex-muscle excitability when males are food-deprived.

Next I looked to see how BK K+ channel/*slo-1* interacts with ERG-like K+ channel/*unc-103*, since both K+ channels are required on food to regulate sex muscle excitability. I made an *unc-103(0); slo-1(lf)* double mutant, and scored males for spicule protraction. I found that double mutant males do not display a significantly higher instance of spicule protraction from *unc-103(0)* single mutants (Table 11, *p* value = 00.43, double to *unc-103(0)*, Fisher's Exact Test). Thus, both *slo-1* and *unc-103* K+ channels are necessary to reduce sex-muscle excitability on food. I then looked at the ability of food-deprivation to suppress *unc-103(0); slo-1(lf)*-induced protraction and found that spicule protraction is unaffected (Table 11). Thus, like *unc-43* and *egl-2*, *slo-1* inhibits sex-muscle excitability when males are food-deprived. Interestingly, it appears that *unc-103* could be partially redundant to *slo-1*, as adding a *slo-1* mutation to the *unc-103(0); slo-1(lf)* does not increase the instance of protraction.

Since BK K+ channels/SLO-1 is working in a similar manner to CaMKII and EAG K+ channels, I asked if they were all part of the same pathway to down-regulate muscle excitability. I created a double mutant between *slo-1(lf)* and *egl-2(0)* and found that the instance of spicule protraction on food was similar to *slo-1(lf)* males (Table 11, *p* value = 0.0816, Fisher's Exact Test). This confirms that *egl-2* plays little role in reducing excitability under standard conditions. *slo-1(lf) egl-2(0)* males display a higher instance of spicule protraction when food is removed than *slo-1(lf)* males alone (Table 11, *p* value = 0.0312, Fisher's Exact Test). This suggests that *egl-2* and *slo-1* work separately to down-regulate muscle excitability when food is scarce.

Table 11. Effects of BK K+ channel/*slo-1* mutants on spicule protraction under well-fed and food deprivation conditions

Genotype[a]	**Food % Protracted (*n*)**	**No Food % Protracted (*n*)**
Wild-type	12 (42)	0 (33)
slo-1(lf)	29 (53)	25 (59)
unc-103(0)	43 (42)	14 (36)[b]
unc-103(0); slo-1(lf)	51 (64)	47 (55)
slo-1(lf) egl-2(0)	39 (33)	48 (40)
slo-1(lf) egl-2(gf)	91 (138)	53 (32)[b]
slo-1(lf) egl-2(0); rgEx253 [$P_{unc\text{-}103E}$*::egl-2(gf)*]	66 (32)	7 (30)[b]
unc-43(sy574)	38 (65)	53 (55)
unc-43(sy574); slo1(lf)	96 (56)	73 (34)[b]

[a]Strains contain *him-5(e1490)*

[b]*p* value < 0.05, Fisher's Exact Test

To confirm that *egl-2* and *slo-1* work separately when males are food-deprived to inhibit spicule protraction, I created a double mutant between *egl-2(n693gf)* and *slo-1(lf)*. *egl-2(n693gf)* does not induce spicule protraction on its own and is able to inhibit *unc-43(sy574)*-induced protraction, indicating it down-regulates sex muscle excitability (Table 11, See Chapter III) [49]. Surprisingly, *slo-1(lf); egl-2(n693gf)* males display a 91% instance of spicule protraction, significantly higher than *slo-1(lf)* on its own (Table 11, p value < 0.0001, Fisher's Exact Test). *slo-1* is broadly expressed in neurons and muscles; mutations in the K+ channel result in an increased pumping rate in the pharynx, the organ *C. elegans* uses to ingest and grind up food [59,104]. On the other hand, *egl-2(n693gf)* decreases the rate of defecation [57]. Combined, these two alleles could cause severe constipation that leads to forced expulsion of the gut contents, damaging the sex muscles and resulting in spicule protraction. To test this hypothesis, I deprived *slo-1(lf); egl-2(n693gf)* males of food and scored spicule protraction. Food deprivation reduced protraction from 91% to 53%, indicating that forced expulsion caused by constipation could be responsible for some, but not all, of the spicule protraction seen in *slo-1(lf); egl-2(n693gf)* males.

I asked what could cause the high instance of spicule protraction in *egl-2(n693gf); slo-1(lf)* males. There are two possibilities: in some manner, EGL-2 is reliant on functional SLO-1 in the sex muscles, or the two alleles cause a synthetic effect upstream of the sex muscles that results in spicule protraction. To test these two hypotheses, the *egl-2(n693gf)* mutation was expressed only in the sex muscles using the $P_{unc\text{-}103E}$ promoter driving *egl-2* cDNA containing the *n693gf* mutation [57,63]. That

way, any synthetic effect occurring upstream of the sex muscles between *egl-2(n693gf)* and *slo-1* can be avoided. I expressed $P_{unc\text{-}103E}$:EGL-2(n693gf) in the sex muscles of *slo-1(lf) egl-2(0)* males and found that 66% of males display spicule protraction on food, a number that is reduced to 7% when the males are removed from food (Table 11). The high instance of spicule protraction seen on food is not higher than *slo-1(lf)* by itself. In addition, since *egl-2(n693gf)* is able to reduce spicule protraction caused by *slo-1(lf)* off food, *egl-2* and *slo-1* likely work in separate pathways to down-regulate sex-muscle excitability off of food.

I next asked if any interaction exists between *slo-1* and *unc-43*. In my experiments, the two K+ channels *egl-2* and *slo-1* appear to work separately, and CaMKII/*unc-43* interacts with *egl-2*, so *unc-43* could interact with *egl-2* and not *slo-1*. On the other hand, *slo-1* has been shown to be downstream of *unc-43* at the presynaptic terminal, and *unc-43* could interact with more than one substrate to regulate spicule protraction [103]. To test for an interaction between *slo-1* and *unc-43*, I made a *unc-43(sy574); slo-1(lf)* double mutant and scored the males for spicule protraction. If the two proteins interact to regulate protraction, than the instance of protraction induced by the double mutant should not be higher than *unc-43(sy574)*-induced protraction. However, if the two proteins are working separately to regulate protraction, than the instance of protraction induced by the double mutant will be higher than either mutation alone. I found that *unc-43(sy574); slo-1(lf)* induces protraction in 96% of males, indicating that the two proteins function separately to inhibit sex muscle excitability (Table 11). Spicule protraction induced by *unc-43(sy574)* is not reduced by food

deprivation and *slo-1(lf)*-induced protraction is only partially reduced by food deprivation. I withheld food from the *unc-43(sy574); slo-1(lf)* double mutant and found that spicule protraction drops from 96% to 73% (Table 11). This is similar to the drop in spicule protraction seen with *unc-103(0); unc-43(sy574)* mutants (See Chapter IV) [49]. The drop in spicule protraction from food to no food can be explained in two ways: (1) *slo-1(lf)*-induced protraction is partially suppress by food deprivation, indicating that pathways are still present to account for the phenotypic reduction, and (2) even if those pathways involved *unc-43*, they could still be functioning in an *unc-43(sy574)* background because *sy574* is a mild loss-of-function *unc-43* allele.

Chapter summary

In this chapter, I identified circuits that reduce the effects of the Ca^{2+} channels that cause male sex-muscle contraction and spicule protraction. There are two Ca^{2+} channels that control sex-muscle contraction, the L-type voltage-gated Ca^{2+} channel (L-VGCC)/EGL-19 and the ryanodine receptor (RyR)/UNC-68, and their effects are regulated differently depending on the feeding state of the animal. When food is plentiful, the effects of EGL-19 Ca^{2+} influx is kept in check by CaMKII/UNC-43, in a pathway separate from the ERG-like K+ channel/UNC-103. UNC-103 attenuates the effects of UNC-68 activity, but UNC-43 does not. In contrast, when the males are deprived of food, UNC-43 can reduce the effects of UNC-68 activity. UNC-43 plays an additional role off food by activating EGL-2 to down-regulate EGL-19 and inhibit sex-

muscle excitability. Parallel to UNC-43/EGL-2, the BK K+ channel/SLO-1 reduces Ca^{2+} influx from EGL-19 when males are both well-fed and food-deprived. In conclusion, *C. elegans* utilizes distinct circuits to reduce muscle sensitivity in the absence of appropriate mating cues and a food source. There are partially redundant mechanisms in place to regulate muscle excitability, suggesting the possibility that one mechanism can be up-regulated to compensate when another is nonfunctional.

CHAPTER VI

SUMMARY OF EXPERIMENTS AND DISCUSSION

Summary of experiments

To identify behavioral regulation at the molecular level I analyzed how the spicule protraction step of male *C. elegans* mating behavior is controlled. I found that different circuits are in place to regulate muscle contraction in response to varying environmental cues, namely the feeding state of the male. Specifically, the kinase CaMKII is active to inhibit sex muscle contraction until appropriate mating cues are received, and this activity is up-regulated in response to food-deprivation to further down-regulate sex muscle excitability. Sex muscle contraction is caused by calcium release from two different channels, L-type voltage-gated Ca^{2+} channels (L-VGCCs) and ryanodine receptors (RyRs), and the functions of these channels are down regulated via different mechanisms. CaMKII works in the muscles and directly interacts with *ether-a-go-go* (EAG) K+ channels to inhibit spicule protraction induced by calcium influx from L-type Ca^{2+} channels. Ryanodine receptor inhibition does not involve EAG K+ channels and involves CaMKII only in the absence of food. Additionally, a BK K+ channel functions in parallel to CaMKII and EAG to inhibit sex muscle contraction in the presence and absence of food. These pathways work in parallel to ERG-like K+ channel inhibition of sex muscle contraction. This study has identified multiple Ca^{2+}-dependent

circuits that coordinate a behavioral response to different feeding states. Such circuits provide a model for behavioral regulation in other organisms.

CaMKII/UNC-43 works in muscles to inhibit sex muscle contraction

I identified a mutation, *sy574*, that induces premature protraction of the male copulatory spicules. The *sy574* lesion is in the *C. elegans* homologue of calcium/calmodulin-dependent protein kinase II (CaMKII), *unc-43*. The regulation of spicule protraction is highly sensitive to disruption as *unc-43(sy574)* induces no other abnormal phenotypes while more severe loss-of-function mutations in *unc-43* lead to many defects. There are two mutations present in the *unc-43(sy574)* allele, one in the substrate recognition site of the enzyme's kinase domain and the other in the self-association domain. However, only the former appears to play a role in *unc-43*'s regulation of sex-muscle excitability as a truncated form of *unc-43*, *unc-43i*, lacking the self-association domain is able to rescue *unc-43(sy574)*-induced spicule protraction. Given the fact that *unc-43(sy574)* appears to only disrupt spicule protraction regulation, and the fact that an early stop codon mutation results in severe pleiotropic defects, it is likely that *unc-43(sy574)* disrupts CaMKII's ability to phosphorylate substrates specific to spicule protraction. *unc-43* is broadly expressed in many tissues and is required in both body-wall and sex muscles to reduce muscle excitability in the spicule protraction circuit. This function parallels that of the ERG-like K+ channel *unc-103*, that is only required in sex muscles to inhibit seizures (Figure 17A).

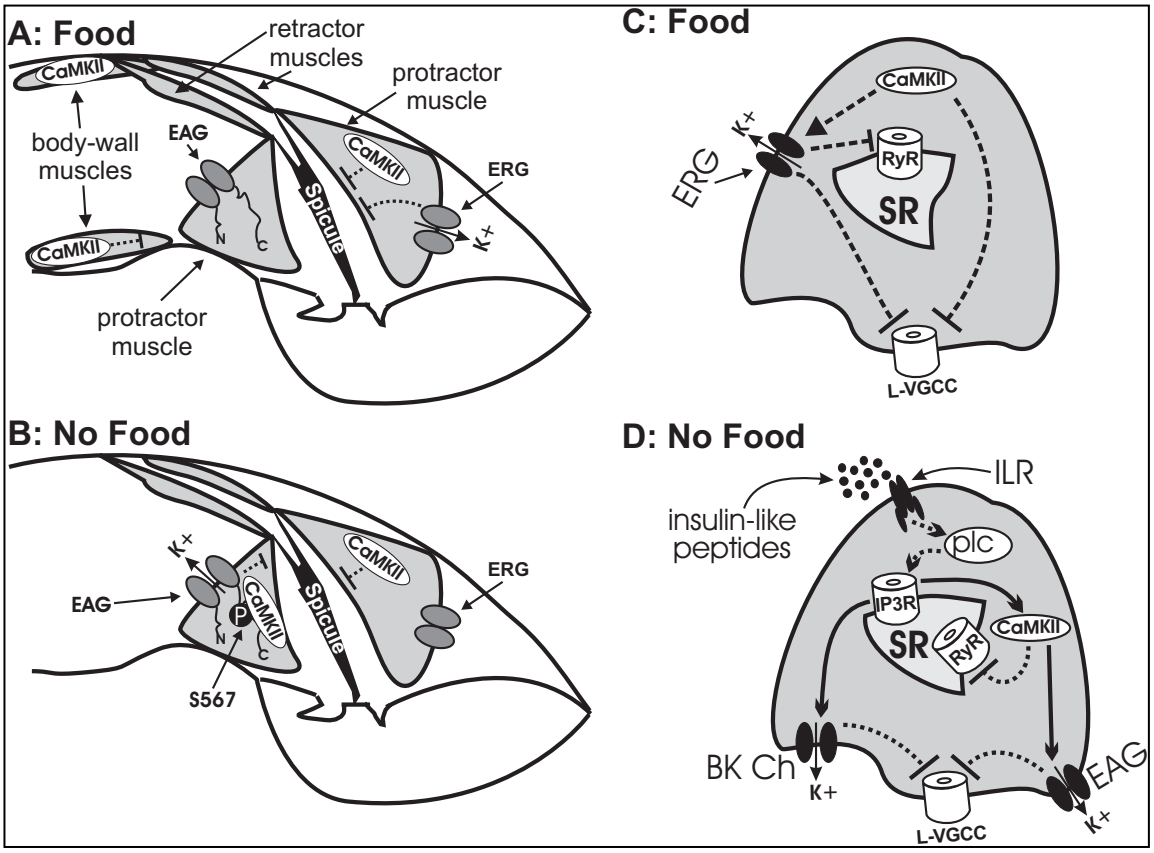

Figure 17. Molecular regulation of spicule protraction behavior in wild-type males. (A)-(B) Diagram of male tail. (A) When the male is food satiated, CaMKII/UNC-43 is active in both the body-wall and sex muscles to down-regulate excitability, while ERG-like K+ channel/UNC-103 is required only in the sex muscles. EAG K+ channel/EGL-2 is inactive. (B) When males are food-deprived, CaMKII activates EAG by phosphorylating S567 to down-regulate excitability. (C)-(D) Diagram of protractor muscle. (C) Under well-fed conditions, CaMKII reduces the effects of calcium influx from L-VGCCs and ERG-like K+ channels attenuates the effects of calcium influx from both L-VGCCs and RyRs. CaMKII also plays a role up-regulating ERG-like K+ channels. (D) When males are food-deprived, insulin-like peptides activate the insulin-like receptor (ILR) DAF-2. This leads to calcium release from IP_3 receptors (IP_3R) that activates CaMKII and BK K+ channel (BK Ch)/SLO-1. BK channels reduce the effects of calcium influx from L-VGCCs. CaMKII reduces the effects of L-VGCCs through EAG K+ channels, and also plays a role in inhibiting the effects of RyRs.

While it is easy to deduce the role of CaMKII/UNC-43 in male sex muscles to inhibit spicule protraction, it is unclear what role UNC-43 is playing in body-wall muscles to regulate the timing of spicule protraction. In the sex muscles, UNC-43 can directly act to inhibit sex-muscle excitability, most likely by both activating proteins that reduce excitability as well as inhibiting proteins that increase excitability. UNC-43 could play a similar role in the body-wall muscles, indicating that they are influencing the activity of the sex muscles. The body-wall muscles are electrically coupled to the sex muscles through the accessory anal depressor muscle that plays no known role in male mating behavior. This signal could in turn be dependent on changing environmental signals that lead to modulation of muscle excitability.

CaMKII/UNC-43 directly interacts with EAG K+ channel/EGL-2 to regulate sex muscle excitability

I identified EAG K+ channel/EGL-2 as a direct down stream target of CaMKII/UNC-43 in the spicule protraction circuit. On food, EGL-2, unlike UNC-43, does not appear to play a role in regulating sex muscle activity under standard conditions (Figure 17A). A null mutation in *egl-2* that results in a large deletion does not result in spontaneous spicule protraction. However, EGL-2 activity is important in the sex muscles to down-regulate muscle excitability in the absence of food (Figure 17B). EGL-2 expression in the sex muscles is sufficient to restore the ability of food deprivation to inhibit *unc-103(0)*-induced spicule protraction in an *unc-103(0); egl-2(0)* background.

Mutating a potential CaMKII site, serine 567, in the c-terminal domain of EGL-2 results in the ability of the mutated channel to inhibit *unc-103(0)*-induced spicule protraction on food. Mutating the serine 567 to phenylalanine could mimic CaMKII phosphorylation and thereby up-regulate channel function.

EGL-2 was determined to be a direct effector of UNC-43 using a yeast two-hybrid assay and *in vitro* protein interaction experiments. Expressing the c-terminus of EGL-2 and the UNC-43 kinase domain in yeast cells resulted in the formation of β-galactosidase, indicating the two proteins interact. In addition, when the potential CaMKII phosphorylation site S567 is mutated, β-galactosidase formation and thus protein interaction is abolished. In conclusion, UNC-43 directly binds EGL-2 and this interaction is dependent upon serine 567 (Figure 17B).

Several circuits exist to inhibit sex muscle excitability under varying environmental conditions

The spicule protraction circuit needs to be tightly regulated to induce protraction when the hermaphrodite vulva has been breached. Premature protraction results in the males' inability to mate. The muscle contraction leading to protraction is caused by calcium influx controlled by L-VGCC and RyRs [47]. To limit the amount of calcium entry into the cytoplasm, K+ channels such as the ERG-like/UNC-103, EAG/EGL-2, and BK/SLO-1 are present, and CaMKII/UNC-43 also plays a role. UNC-103 reduces the effects of calcium influx from both sources, while UNC-43 is primarily involved in

reducing the effects of L-VGCCs under standard conditions; the downstream effectors targeted by UNC-43 in this situation have yet to be identified (Figure 17C) [12]. While analysis of double mutant *unc-103(0); unc-43(sy574)* males indicates the two genes function in separate pathways in the spicule protraction circuit, an UNC-103-dependent component of UNC-43-mediated protraction suppression exists. On one hand, nearly all *unc-103(0); unc-43(sy574)* males display spontaneous protraction, indicating they function in separate pathways to regulate the spicule circuit. On the other hand, UNC-103 is required for UNC-43-mediated response to ACh agonists, and a nonsense mutation in *unc-43* results in nearly all males displaying inappropriate protraction. This suggests a role for UNC-43 in UNC-103-mediated suppression of sex-muscle excitability.

Additional mechanisms are in place to further suppress spicule protraction under food deprivation (Figure 17B,D). Both UNC-43 and EGL-2 decrease sex-muscle excitability by reducing the effects of calcium influx from L-VGCCs, as lack of these functional proteins results in the inability of food deprivation to suppress *unc-103(0)*-induced spicule protraction. Interestingly, removing males from a food source increases the instance of protraction in a mutant allele of *unc-43*, *sy574*. This suggests an excitatory signal is activated by food deprivation and UNC-43 functions as a negative regulator of this signal. The excitatory signal activated by food deprivation could come from sarcoplasmic reticulum-located ryanodine receptors (RyRs)/UNC-68. Since UNC-43 reduces calcium influx into the cell from UNC-68 under food deprivation conditions, calcium released by UNC-68 could increase UNC-43 activity, ultimately resulting in

reduced sex-muscle excitability. The mechanism by which UNC-43 down-regulates UNC-68 is unknown, but UNC-68 could be a direct target of UNC-43, where phosphorylation of UNC-68 by the kinase inhibits channel function. UNC-68 possesses a potential CaMKII phosphorylation site at S3059, which is in the same region on the RyR as the human CaMKII site S2814 [105,106]. Whether CaMKII phosphorylates RyRs to reduce or increase channel function in mammalian systems is controversial [107-110], though if UNC-43 phosphorylates UNC-68 in the spicule protraction muscles, it is doing so to reduce channel function.

My research focused on the molecular circuits inside the male sex muscles that result in reduced excitability both on and off food. However, how these circuits are activated remains a question. Since two of the proteins involved, CaMKII/UNC-43 and BK K+ channel/SLO-1, are activated by calcium, I hypothesize that a calcium signal is initiated. Part of the upstream pathway resulting in release of a calcium signal was identified by a fellow graduate student, Todd Gruninger [75]. He found that loss of the food signal leads to activation of the AWC olfactory neurons; these neurons have exposed processes at the tip of the head to sense the environment. AWC could then activate downstream neurons that release insulin-like peptides, as the insulin-like receptor DAF-2 is involved in the males' sex muscle response to the food-deprivation signal. DAF-2 is proposed to activate phospholipase-γ (PLC-γ), which in turn causes calcium to be released from inositol 1,4,5-trisphosphate receptors (IP_3Rs) (Figure 17D). In mammalian systems, PLC-γ increases the intracellular calcium that activates CaMKII and is activated by insulin-like growth factor [111-113]. It is the calcium released from

intracellular stores via IP_3Rs that is hypothesized to down regulate muscle excitability through UNC-43/EGL-2 and SLO-1 (Figure 16D) [75].

Downstream synaptic connections of AWC include the interneurons AIY and AIB that are involved in mediating response to food [114,115]. AIY and AIB act as an opposing neuron pair, as AIY inhibits a systematic response to food deprivation and AIB actives a systematic response [114]. Therefore, the activation of AWC caused by a removal of the food signal could result in activation of AIB and inhibition of AIY that leads to decreased muscle excitability.

Mechanisms of behavior response to environment stimuli identified in *C. elegans* provide insight into behavioral regulation in other organisms

A period of food deprivation results in many changes in an organism, including an increase in life span, resistance to stress, and reducing the instance of epileptic seizures [116-121]. These changes have been recorded in organisms from *C. elegans* to mice, and the molecular circuits underlying these changes are just beginning to be explored [122,123]. Because food deprivation has systemic effects on an organism, identifying the underlying mechanisms by which food deprivation produces those effects becomes essential for targeting treatment while avoiding undesirable side effects. My work has identified existing parallel mechanisms that are activated by a period of food deprivation whose end result can reduce cell excitability and, by extension, seizures. Though this work was done in *C. elegans*, it provides focus for researchers in less

genetically tractable organisms. In the area of food deprivation's reduction of seizure susceptibility, much work remains to be done on the mechanisms involved in dietary restriction's effect on seizures [124,125]. Research in *C. elegans* can identify molecules and circuits that potentially regulate similar behavioral responses in other organisms.

My work identifies CaMKII as a mechanism for changing behavioral output in response to food deprivation [49]. Few studies have explored the activity of CaMKII during food deprivation, as most research centers on its role in learning and memory and the underlying neuronal changes referred to as long term potentiation (LTP) [77,78,126]. However, since CaMKII was identified as an important kinase in neurons, its potential role in detrimental conditions that affect neuronal function, such as epilepsy and aging, has been examined [127,128]. Reduced CaMKII function is implicated in seizure disorders, as well as impaired LTP during aging [127,128]. This raises the possibility that CaMKII provides a mechanistic link between the positive effects of food deprivation that reduce the instance of seizures and effects of aging. My work supports the existence of this possibility, as I show a direct link between CaMKII and reducing cell excitability in response to food deprivation. While many more studies need to be done in vertebrates to discover the mechanisms relating food deprivation to seizures, my research provides directionality of study for exploring the mechanistic link between CaMKII, food deprivation, seizures, LTP, and aging.

There are many difficulties inherent in piecing apart the many functions of a protein involved in a variety of cellular functions in addition to behavior. Broadly disrupting the function of CaMKII in *C. elegans* using an allele that creates an early

nonsense mutation results in systemic defects in behavior [76]. In addition, impaired CaMKII function has been shown in other organisms to disrupt everything at a cellular level from gene transcription to protein activity [84,129]. Since CaMKII can have multiple functions in the same cell, this makes exploring its role in regulating specific behaviors difficult even in *C. elegans*. Forward genetic screens allow for the identification of multiple alleles of a gene that have different effects on protein function, facilitating exploration of the protein's role in different molecular processes. I utilized two CaMKII alleles identified separately, one by Dr. L. Rene Garcia at Cal Tech in the late 1990s and another by Sydney Brenner in the 1960s, to demonstrate the role of CaMKII in regulating male mating behavior under well-fed and food deprivation conditions [4,12]. Without the availability of these unique alleles, I would not have identified such a thorough circuit that CaMKII uses to regulate behavior. Such forward genetics screens are difficult to perform in more complex organisms. However, once proteins and their potential functions have been identified, techniques exist that allow for targeted disruption of protein function. Thus, research into behavioral regulation and environmental response performed on *C. elegans* provides insights for how behaviors are controlled and sensory cues integrated in more complex organisms.

Future directions

In this work, I identified how individual proteins, namely CaMKII and voltage-gated K+ channels, function in specific circuits and can be modified to produce changes

in behavioral regulation. However, these are not the only molecules involved in controlling male mating behavior, as aspects of the circuits down-regulating cell excitability under both well-fed and food deprivation conditions have yet to be identified. While the EAG K+ channel EGL-2 appears to be an effector of CaMKII/UNC-43 when food is scarce, the question of what CaMKII phosphorylates to reduce sex muscle excitability when males are satiated remains a question. Additionally, CaMKII is a calcium-activated kinase and the calcium sources activating the kinase have yet to be definitively established. Finally, I propose an additional method to identify other proteins regulating male mating behavior.

Identifying the direct effector(s) of CaMKII/UNC-43 in the spicule protraction circuit

One of the biggest missing links of CaMKII/UNC-43 regulation of spicule protraction is the identity of direct UNC-43 effectors under standard conditions in the body-wall and sex muscles that inhibit premature protraction. A mutagenesis screen was performed on worms carrying a gain-of-function *unc-43* allele in an attempt to address this question. The *unc-43(gf)* allele does not induce spicule protraction but impairs movement in both sexes and egg-laying in hermaphrodites [53]. Previous work looked for down-stream effectors of *unc-43* that control movement, as *unc-43(gf)* worms were mutagenized to look for improved locomotion. These screens identified members of G-protein signaling networks [130]. In my screen, mutagenized animals also carried the

unc-103(0) allele, to sensitize the spicule protraction circuit, since *unc-103* is activated by *unc-43(gf)* to inhibit sex-muscle excitability [49]. Mutations were isolated that induced spicule protraction but did not affect the *unc-43(gf)* movement and egg-laying phenotypes to avoid identifying the same down-stream molecules as the previously performed *unc-43(gf)* mutant screen. These mutations were analyzed to identify effectors that are only involved in spicule protraction. *unc-103(0); unc-43(gf)* worms are very ill, and I found that it was difficult to obtain many mutagenized lines. Few of the surviving lines displayed spicule protraction and the one line analyzed was a revertant in the *unc-43* gene that affected splicing.

To identify downstream effectors of *unc-43* in regulating spicule protraction and to avoid the problems presented by working with *unc-103(0); unc-43(gf)* strains, an *unc-103(0)* strain with *unc-43(gf)* expressed only in the sex muscles via the $P_{unc\text{-}103E}$ promoter can be used for mutagenesis instead. Without the systemic effects of the *unc-43(gf)* allele, and since *unc-103(0)* displays no systemic effects, the survival of mutagenized lines should increase. Mutations that induce spicule protraction in an *unc-103(0); sex muscle unc-43(gf)* background can then be isolated and the molecular nature of the lesion identified. In *C. elegans*, mapping mutations is carried out utilizing single nucleotide polymorphisms (SNPs) that exist between the standard lab strain, N2, and a Hawaii isolate, CB4856 [60]. The existence of these SNPs allows for rapid, though labor-intensive, mapping of genetic loci. This procedure has recently been refined to make rapid identification of the genetic loci of mutations easier, allowing for a larger number of mutations to be identified in a shorter period of time [131]. Thus, if the

mutagenesis screen on *unc-103(0); unc-43(gf)* animals is successful, the molecular location of the lesions causing spicule protraction can be identified quickly, greatly extending our knowledge of proteins involved in regulating spicule protraction.

The biggest caveat for using an *unc-103(0); sex muscle unc-43(gf)* line is that *unc-43* is also required in the body-wall muscles to inhibit sex muscle contraction. Expressing *unc-43(gf)* in the sex muscles alone might not enable the identification of a direct target that is required in the body-wall muscles as well.

Another way to do the mutagenesis screen is to use *unc-43(gf); egl-2(0)* hermaphrodites. Like *unc-103*, *egl-2* is down-stream of *unc-43* regulation of sex muscle contraction, and removing *egl-2* would increase the likelihood of another mutation inducing spicule protraction in an *unc-43(gf)* background [49]. Mutagenized *unc-43(gf); egl-2(0)* lines might have a higher survival rate than *unc-43(gf); unc-103(0)* lines, since *egl-2* is not as widely expressed as *unc-103* and does not appear to affect as many systems [58,63]. For example, *unc-103(gf)* hermaphrodites display both egg-laying and movement defects, while *egl-2(gf)* hermaphrodites only display egg-laying defects [57,132]. Therefore, removing *unc-103* might be more detrimental than removing *egl-2*. If mutagenized *unc-43(gf); egl-2(0)* lines are just as sick as mutagenized *unc-43(gf); unc-103(0)* lines, *unc-43(gf)* can be limited to sex-muscle expression in *egl-2(0)* worms. However, I favor mutagenesis of *unc-103(0); sex muscle unc-43(gf)* hermaphrodites because removing *unc-103* sensitizes the sex muscles to a degree that *egl-2* does not, since *unc-103* plays a more active role under standard conditions inhibiting spicule protraction than *egl-2*.

The possibility exists that CaMKII/*unc-43(sy574)* males display spontaneous spicule protraction due to reduced ability of UNC-43 to phosphorylate itself. CaMKII can be phosphorylated by other CaMKII enzymes, most likely by those that are in the same 8 or 12 member complex formed by association of the self-association domains [80,81]. Self-phosphorylation allows the kinase to remain active when the initial activating Ca^{2+} signal has been removed, and it also allows the kinase to increase its response to repeated influxes of Ca^{2+} [133]. Thus, while *unc-43(sy574)* might still be able to phosphorylate most substrates, its impaired ability to phosphorylate itself would hinder its response to repeated Ca^{2+} signals. This would allow the Ca^{2+} signal *unc-43* is responsible for reducing the effects of to build up in the cell unopposed, leading to a higher level of Ca^{2+} able to cause the permanent sex-muscle contraction that leads to spicule protraction. However, while this might be the case in *unc-43(sy574)* mutants, UNC-43 still must have direct molecular targets that down-regulate muscle excitability. Therefore, the mutagenesis screen to identify down-stream targets is still a worthwhile endeavor. However, the idea that *unc-43(sy574)* reduces self-phosphorylization raises the possibility that the downstream effectors of *unc-43* might not be specific to the circuit regulating spicule protraction; the system might simply be more sensitive to the level of CaMKII phosphorylation.

Identifying the Ca^{2+} sources that activate CaMKII/UNC-43 under well-fed and food deprivation conditions

CaMKII/UNC-43 is activated by Ca^{2+} bound to calmodulin, raising the question of the origins of the Ca^{2+} source. In his paper entitled "Sensory perception of food and insulin-like signals influence seizure susceptibility," Todd Gruninger suggests a Ca^{2+} signal initiated by the insulin-like receptor *daf-2* and working through phospholipase C-γ (PLC-γ) activates UNC-43 and EAG K+ channel/EGL-2 to inhibit sex muscle excitability under food deprivation conditions [75]. PLC-γ is downstream of insulin receptor activation in mammalian systems and activates CaMKII in neuronal cells [111-113]. In addition, loss of PLC-γ function inhibits the ability of food deprivation to inhibit *unc-103(0)*-induced spicule protraction [75]. PLC-γ cleaves PIP_2 to create the second messenger $InsP_3$ that activates calcium-releasing IP_3 receptors (IP_3Rs) located on the endoplasmic reticulum. It is this Ca^{2+} that is then hypothesized to activate UNC-43.

To test this idea, DAF-2 function can be reduced in spicule protraction mutants via RNA interference (RNAi). RNAi works by reducing the amount of messenger RNA of the target gene [8]. Worms can be fed *E. coli* containing double-stranded RNA of the gene to be knocked down. This allows for time-specific reduction of the gene. To reduce the amount of DAF-2 present in the cell, a mutation in the RNAi pathway has to be present in the mutant background, to allow more complete penetration of the *daf-2* RNAi. Reducing *daf-2* function in *unc-103(0); unc-43(sy574)* and *slo-1(lf)* males will allow me to test if they maintain well-fed levels of spicule protraction under food

deprivation conditions. If so, this would indicate that signaling from the DAF-2 insulin-like receptor is necessary to activate mechanisms present in these worms to down-regulate muscle excitability under food deprivation conditions.

The calcium source activating CaMKII under well-fed conditions has yet to be identified. One source of calcium could be produced by signaling through the muscarinic acetylcholine receptor GAR-3-$G\alpha_q$ pathway. GAR-3 has been shown to sensitize the sex-muscle circuit's response to the vulva, as males lacking the GAR-3 receptor have difficulty maintaining proper positioning of their tails over the hermaphrodite vulva. Wild-type GAR-3 responds to the neurotransmitter acetylcholine, and the receptor can be activated by the agonist oxotremerine M (Oxo M). Oxo M induces mating-like behavior in males while having no affect on hermaphrodites; this phenotype is abolished when the GAR-3 receptor is removed. The low level of ACh signaling that activates GAR-3 is hypothesized to be present in the circuit controlling mating in wild-type males and could cause an undesired increase in calcium if not controlled properly [61]. CaMKII, as a calcium-sensitive molecule responsible for decreasing sex-muscle excitability, could be a means by which GAR-3 signaling is kept in check. To test this hypothesis, signaling from the GAR-3 pathway can be interrupted to reduce spicule protraction caused by disrupting the CaMKII gene *unc-43*. Double mutants consisting of loss-of-function *gar-3* and *unc-43* alleles can be generated and scored for the instance of spontaneous spicule protraction. Additionally, the timing of spicule protraction can also be determined in *unc-43(lf); gar-3(lf)* males, by scoring protraction every hour after a male becomes an adult, to see if *unc-43(lf)*-induced

protraction is delayed. To further analyze the role of *unc-43* in regulating excitability generated by GAR-3, the affects of the GAR-3 specific agonist Oxo M on *unc-43* loss-of-function males can be determined. Disrupting *unc-43* function could sensitize the muscles to Oxo M. While signaling from GAR-3 that sensitizes the spicule circuit for mating is one possible source of the calcium activating CaMKII under standard conditions, many other signaling inputs are present as well. Therefore, inhibiting GAR-3 signaling could have a negligible over effect on spicule protraction caused by impaired CaMKII function.

Identifying components that can improve mating performance

By disrupting protein function through mutagenesis, researchers hope to then identify what the wild-type protein does by examining behavioral deficiencies in mutants. In this way, ERG-like K+ channel/*unc-103* and CaMKII/*unc-43* were identified as proteins that regulate the timing of sex muscle contraction [12,49]. However, mutant screens looking for deficiencies in behavior can also identify non-specific muscle and neuronal proteins necessary for muscle contraction and signal transduction. To avoid this problem and identify more components regulating male mating behavior, I propose to identify mutants that are more successful at siring progeny than the standard N2 strain.

The ability to obtain such mutants is illustrated by the difference in mating ability between two *C. elegans* strains, N2 and CB4856. When N2 males are given twenty

minutes to mate to paralyzed hermaphrodites, 49% (n = 240) are able to sire progeny. In contrast, 74% of CB4856 males are able to sire progeny (n = 270, p value < 0.0001 compared to N2, Fisher's Exact Test, see Appendix F), indicating that the CB4856 strain mates with a much greater efficiency than N2. The difference in strain mating has been reported elsewhere, as over a period of 6 days CB4856 males have more mates and produce more offspring than N2 males [134]. This indicates that the differing ability to sire progeny is not a particular phenotype of the N2 and CB4856 lines I have used.

Since there naturally exists a possible allele difference between N2 and CB4856, I can first map and characterize this mutation. Many single nucleotide polymorphisms exist between the two strains, making mapping easier [60]. In addition, recent techniques have been developed to reduce the amount of work and time necessary for SNP mapping [131]. Mating can be watched and analyzed to see if there is a particular step that CB4856 males are better at, as can muscle response to various acetylcholine agonists. Previous work has indicated that CB4856 males mate more frequently than N2 males, though there is no difference in the amount of time until the male first contacts the hermaphrodite, time until spicule insertion, or number of successful spicule insertions [134]. I have data that supports the idea that CB4856 males mate more frequently than N2 males, though my data further indicates that CB4856 are more persistent in mating than N2 (see Appendix F). Acetylcholine is the predominant neurotransmitter used in the sex muscle circuit, causing contraction of the spicule muscles during mating [47]. Acetylcholine has also been proposed to increase the sensitivity of the spicule circuit prior to mating, indicating that subtle variations in

signaling can affect mating behavior [61]. In this manner, the molecular nature and specific behavioral consequences of improved mating can be identified.

In additional to utilizing SNP mapping to identify the location of the molecular difference resulting in improved mating, previously identified allelic differences between N2 and CB4856 can be analyzed for their role in mating behavior. There exist two changes with known consequences in *C. elegans*. One is in the *plg-1* gene, which encodes a glycoprotein that is the major structural component of the copulatory plug. The males of some strains of *C. elegans* are able to place copulatory plugs over the hermaphrodite vulva to prevent other males from mating. CB4856 males are able to create plugs, while N2 males are not. N2 males have a retrotransposon inserted into an exon of *plg-1*, rendering the gene inactive [135]. However, this does not appear to have any functional consequences for the mating behavior itself, and therefore it is unlikely the mating differences that exist between CB4856 and N2 are a result of *plg-1* [135]. The other allelic difference between the two strains is in the *npr-1* gene. *npr-1* encodes a G-protein-coupled neuropeptide receptor similar to the mammalian neuropeptide Y receptor that is involved in modulating a number of behaviors [136]. The allelic difference between the two strains, a valine at amino acid 215 in N2 and a phenylalanine at the same position in CB4856, has behavioral consequences. The 215V N2 allele is dominant to the 215F allele, as the 215V receptor has increased activity that promotes "social feeding," which indicates groups of worms feeding together at the edge of a bacterial lawn [136]. This increased social behavior is driven by the desire to be in areas of lower oxygen concentration and results in reduced resistance to pathogens due to

remaining on the pathogen in a group, as opposed to the more solitary N2 animals [137-139]. Since variation in *npr-1* has already been shown to have subtle effects on behavior, it is a candidate for the differences between CB4856 and N2 male mating success. Since CB4856 have lower functioning *npr-1* receptors and increased ability to sire progeny, it would be interesting to determine how reducing the neuropeptide Y-like receptor increases mating behavior. Loss of *npr-1* function results in increased social behavior, which could in turn promote more frequent mating. While in the mating assay I employ only one male is mated to one hermaphrodite for a period of 20 minutes, negating any immediate group component, the males are raised together on plates containing *E. coli* prior to the mating experiment. When any group of males is isolated from potential mates, be it N2 or CB4856, they turn to attempting to mate with one another, repeatedly scanning along male cuticles searching for the vulva. They will even attempt to mate with themselves. During this time, CB4856 males could spend more time in contact with one another (and themselves) than N2 males, increasing the activity of the male mating circuit and making these animals more likely to mate. Previous work as identified a $G\alpha_q$-mediated network that up-regulates vulva response in the spicule circuit [61]. *npr-1* or another unidentified protein could play a role in mediating the activity of this network, modulating the male's drive to mate. In conclusion, studying male mating differences between naturally occurring isolates of *C. elegans* and identifying their underlying causes can give insights into behavioral regulation.

Conclusion

Studying the regulation of the spicule protraction step of *C. elegans* male mating behavior has elucidated important mechanisms by which behavioral control is achieved. In particular, responses to the environment can be coordinated through up-regulation of specific pathways. Identifying the molecular nature of these mechanisms allows them to be targeted for manipulation to compensate for the loss of other components in the circuit. In addition, given the controversies surrounding some of these proteins studied in mammalian systems, *C. elegans* offers an avenue for insight into their function as well as a breadth and depth of investigation that is not always possible in other organisms. Future studies of coordinated environmental responses will expand the knowledge of how specificity is achieved in behavioral regulation.

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APPENDIX A

SUPPLEMENTARY TABLES

Table A-1: Primers

Primer name	Primer sequence
U43cDNAstart	CGCGCGGCCGCGCGCGCGGGTACCATGATGAACGCAAGCACCAAGTTT
U43cDNA3UTR	TTTATTTTAAGATTTTTTTGTTGCATTTGTCATCATATC
fpbl12a	CCAAAATCATCAACACAAAGAAGCTATCC
pbl12ar	CAGCAAACTCGAGGCCCGTGGT
FYFPEAG	CGCGCGCGGCCGATGAGTAAAGGAGAAGAACTTTTCACTGGAG
YFPRKPN	CGCGCGGGTACCTTTGTATAGTTCATCCATGCCATGTGTAATC
Fpbl33ssm	ATGATGAACGCAAGCACCAAGTTTAGTGAC
Pbl33ssmr	CGGCCATCACCACTTTGTACAAGAAAGCTG
Fpbl333utr	AAGAACCTTCTTTATGCCTATTTTTCTCTTCGTTTCC
Rpbl33stop	CGTGTTTCAGCTATCCACCACCTTTTTCCG
attb1aex-3p	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGGAACACGCTAATTACTCGTGTGTGCTAC
attb2aex-3p	GGGGACCACTTTGTACAAGAAAGCTGGGTTGGTGCAAGCCTGGACAAATTTTGAAAAAG
attb1lev11prof	GGGGACAAGTTTGTACAAAAAAGCAGGCTCACAGCGATGATGTGTCATGGCTTCC
attb2lev11pror	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTTGGTAGTTTGTTGTTGTGTTGAAACACACGGAGTATCGAC
attb1tntproup	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCAAAATTAGCTCAATTGATCAAATAACTG
attb2tnt4prodown	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTGATTGGTGAATTGGTTGTAAAAAAAACCCCT
acr-8ATTB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTATTGGACGAGGTGGTCCCATTCCCACTAAGT
acr-8ATTB2	GGGGACCACTTTGTACAAGAAAGCTGGGTGGTATTGTCGGTTTCATCGCGTGGTGAGGAG
unc43att2bcsf	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGATGAACGCAAGCACCAAGTTT

Table A-1 Continued	
Primer name	**Primer sequence**
unc43att2bc3r	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTATTTTAAG ATTTTTTTGTTGCATTTGTC
att1gtl1p	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGCAAAATA CTTTGGCGCCACAGATGC
gtl1patt2	GGGGACCACTTTGTACAAGAAAGCTGGGTTCAATTAAAAC TTTTTGCTCTGAACTTTGG
fegl2n698gf	CTCCGCACTTTTGTATGTTGCAATTTTTGGAC
Regl2698gf	ATGATCATCATGCAGACTCCGAATATCTTCTC
Fegl2n904	GTTGTTTTCGTCTTGCATTTGACGGGTGT
Egl2n904r	TGTGCTCATTGAAAACCTTTCGATTCAAGTGAAC
Fegl2rsvs	AGGAGCTGGTGGAGTGAFCAAC
Egl2rsvsr	TGCGACAGGCGCCGCGTCAAGT
fEgl2end	CGTCCTCCGGCAAGAACACGGATATGA
Fdelpasegl2	CAGGTTATCAAGGTTATGAACCTTGGTGG
Delpasegl2R	AAGTCCACGTTTCCCAACCGGCAT
func43iecor1	GCGCGAATTCATGATGAACGCAAGCACCAAGTTTAGT
unc43iecor1r	GCGCGAATTCTCACTCGTTACCTTTTTCCGAATCGTTG
sy574ar	TTCACTATCGTTCACTTCGATTGCAAGACC
fsy574a	GCATGGCACGAATTTGCTGGAACTCC
Ff13b123	GACCTGATCTCAACTTCACAGTCGT
F13B123R	GCCCGCCTTGTGTCCAG
FR09H106	CCAGATTCACGCAAGACAATGG
R09H106R	CAGTCGATTTCCGGCGCGCTTACAATC
Fc43f96	ATGATGCGGTGCTGCATTGT
C43f96r	CAAACTTCTCGACAGACGGTGAGTT
FUnc43inact	CGGTATTGTTCACAGAAACTTGAAGCCAG
Unc43inactr	TTAGAGTGGCAATAAGCAATCGATTCGAG
Unc43irev	AATCCATGGGACTTTCAAGGCCTGATC
ForPGBKT7	GAATTCCCGGGGATCCGTCGAC
fegl2cterm	CGCGGAATTCCAACAAATGACATCCAGTACTGTGAGATAT CA
egl2cterm2r	CGCGCGATCGTCATATCCGTGTTCTTGTCGGAGGAC
Ftax6	CTCTGGCAATTTCCAAGCTTGCA
tax6r	ACCGATTGATTTTTGTTGCTTTTCCCTTGC
Nwgpa7f	GAAGAGTGGTGCTGCGTAGTCA
Gpa7nwr	GGATTTCTGTGGAATGATGTACCCG
Fcal4	GTAACTAGAAGACGATCTACGTCTTGG

Table A-1 Continued	
Primer name	**Primer sequence**
GADT7ssmutr	GAATTCACTGGCCTCCATGGCCAT
Cal4r	AGTAGTTTCCGCTCCAGCACAT
FGADT7	GGATCCATCGAGCTCGAGCTGC
egl2hind3r	CGCGAAGCTTTCATATCCGTGTTCTTGTCGGAGGAC
Fegl2n904	GTTGTTTTCGTCTTGCATTTGACGGGTGT
Egl2n904r	TGTGCTCATTGAAAACCTTTCGATTCAAGTGAAC
func43pro	GTGTGTATGTCTGTGAATTGGCTCTCA
u43prcfp	TTGGCCAATCCCGGGGATCCGGCTAATTGCTGAAGATAGT GCTGATCTACAGTT
cfpfu43p	ACTATCTTCAGCAATTAGCCGGATCCCCGGGATTGGCCAA AG
u54rev	GGGCCCGTACGGCCGACTAGTAGG

Table A-2: Plasmids	
Plasmid	**Relevant Contents**
pBL12b	UNC-43g cDNA with point mutation
pBL13	point mutation in pBL12b corrected
pBL14	YFP:UNC-43g
pBL33	Gateway RFC A:YFP:UNC-43g
pBL33-YFP	Gateway RFC A:UNC-43g
pBL33-self asso	Gateway RFC A:YFP:UNC-43i
pDG15aex-3	Gateway entry clone containing *aex-3* promoter
pLR22	Gateway entry clone containing *lev-11* body-wall muscle promoter
pLR25	Gateway entry clone containing *tnt-4* promoter
pLR92	Gateway entry clone containing *acr-8* promoter
pLR21	Gateway entry clone containing *unc-103*E promoter
pBL70	P_{aex-3}:UNC-43g
pBL71	$P_{unc-103E}$:UNC-43g
pBL69	P_{lev-11}:UNC-43g
pBL72	P_{tnt-4}:UNC-43g
pBL80	P_{acr-8}:UNC-43g
pBL68	P_{lev-11}:YFP:UNC-43i
pBL75	$P_{unc-103E}$:YFP:UNC-43i
pLR99	P_{acr-8}:YFP
pTG14	P_{hsp-16}:Gateway RFC C.1
pBL54	Gateway entry clone containing UNC-43g
pBL58	P_{hsp-16}:UNC-43g
pBL63	Gateway entry clone containing *gtl-1* promoter
pGW77C	Gateway RFC C.1:CFP
pBL66	P_{gtl-1}:CFP
pTG44	$P_{unc-103E}$:EGL-2(+)
pBL111	$P_{unc-103E}$:EGL-2(n693gf)
pBL109	$P_{unc-103E}$:EGL-2(n904)
pBL110	$P_{unc-103E}$:EGL-2(RSVS)
pBL108	$P_{unc-103E}$:EGL-2(lacking aa 891-949)
pBL122	$P_{unc-103E}$:EGL-2(lacking aa 9-185)
pBL81	UNC-43 kinase + inhibitory domains placed in yeast expression vector pGBKT7, c-Myc epitope tag
pBL85	pBL81 with inactive kinase domain by D135N mutation

Table A-2 Continued	
Plasmid	**Relevant contents**
pBL88	UNC-43 kinase domain with D135N mutation
pBL93	*egl-2* c-terminus in yeast expression vector pGADT7
pBL99	*egl-2(+)* c-terminus in *E. coli* expression vector pMal-C2
pBL114	*egl-2(n904)* c-terminus in *E. coli* expression vector pMal-C2
pBL120	UNC-43g:GST in *E. coli* expression vector pGEX-3T
pBL123	UNC-43i:GST in *E. coli* expression vector pGEX-3T

APPENDIX B

CaMKII INHIBITS SPC NEURON AND ANAL DEPRESSOR MUSCLE SIGNALING

Removing the SPC motor neuron and anal depressor muscle from *unc-43* mutants reduces spicule protraction

The study of *C. elegans* allows researchers to remove individual cells or groups of cells to determine their function. This has facilitated identification of the circuit controlling spicule protraction [29,47]. In the male sex circuit, spicule protraction is controlled by the SPC motor neuron that uses the neurotransmitter acetylcholine to signal the protractor muscles to contract. Attached to the protractor muscles is the anal depressor muscle whose function has not yet been elucidated. However, in cell ablation studies where a laser was used to perform microsurgery on individual males and remove a cell or groups of cells, the anal depressor was identified as being involved in signaling the protractor muscles to contract [12]. When the function of the ERG-like K+ channel *unc-103* is disrupted with the *sy557* allele, both the SPC motor neuron and anal depressor muscle have to be ablated to reduce spicule protraction. Therefore, there is signaling originating in both these cells that increases sex muscle excitability *unc-103* is responsible for controlling. I wanted to see if signaling from the SPC motor neuron and anal depressor could induce excitability that CaMKII/*unc-43* was required to reduce. I ablated the SPC neuron and anal depressor separately and together in *unc-43(sy574)* and

unc-43(n1186) males. I used both alleles since *unc-43(sy574)* is a partial loss-of-function and *unc-43(n1186)* contains an early stop codon, reducing *unc-43* function to a much greater extent [49,63]. I found that ablating either the SPC neuron or the anal depressor in *unc-43(sy574)* males reduced spicule protraction while ablating them in combination had an even great effect (Figure B-1). In contrast, both the SPC neuron and anal depressor had to be removed from *unc-43(n1186)* males to reduce protraction (Figure B-1). Therefore, *unc-43* is required to inhibit excitation signals from both cells.

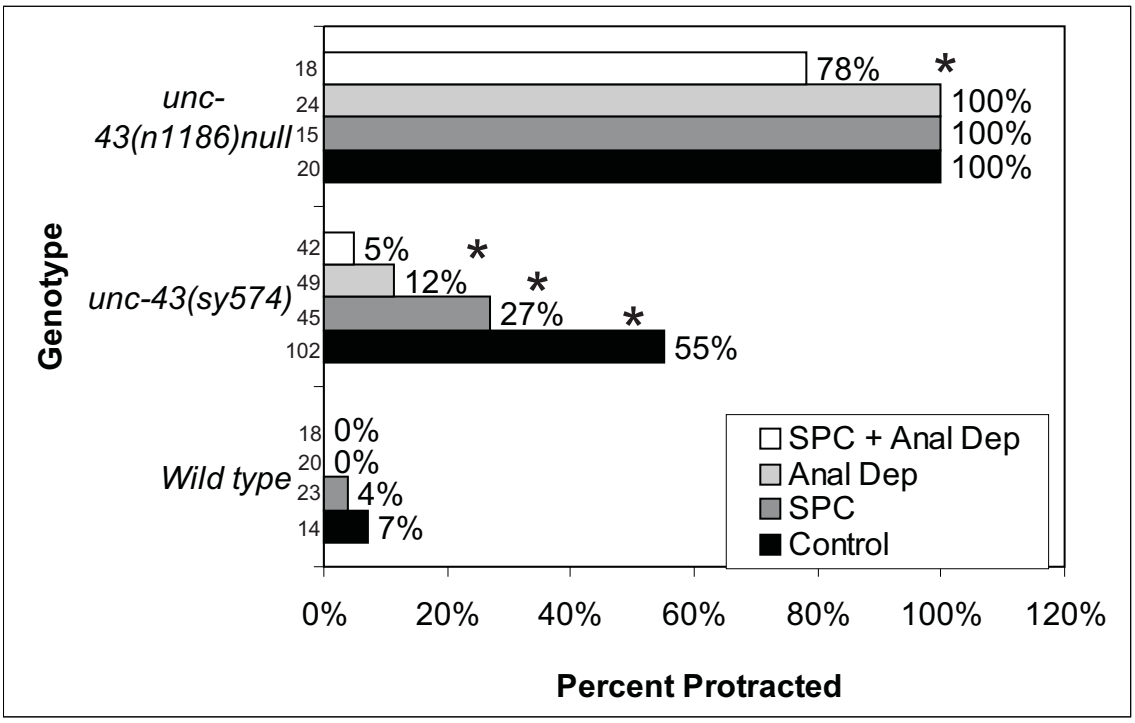

Figure B-1. Ablating the SPC motor neuron and anal depressor muscle. Affect of ablating cells in the spicule protraction circuit on *unc-43* mutants. *x* axis is the percentage of protraction constitutive (prc) males, *y* axis is the genotype of the ablated males. The *n* for each value is indicated next to the left of each bar.

APPENDIX C

HOW *UNC-103(SY557)* POINT MUTATIONS INFLUENCE SPICULE PROTRACTION

unc-103(sy557B) expressed in sex muscles and neurons induces premature spicule protraction

The *unc-103(sy557)* allele of the ERG-like K+ channel results in a significantly higher instance of spicule protraction than deleting the *unc-103* gene, raising the question of how the two point mutations present in *unc-103(sy557)* affect channel function [12]. The fact that *unc-103(sy557)* induces a higher rate of spicule protraction than *unc-103(0)* indicates that the point mutations result in a channel able to interfere with non-*unc-103*-mediated spicule protraction regulation. Members of the ERG K+ channel family contain six transmembrane domains, the last two of which form the pore region. *sy557A* changes a histidine to asparagine and is located in the linker region between transmembrane domains two and three (Figure C-1). *sy557B* changes a tryptophan to an arginine in transmembrane domain five and could directly affect the open probability of the channel (Figure C-2). To determine the effect of the mutations on channel function, a plasmid containing *unc-103* genomic DNA was mutated. The mutated *unc-103* genomic DNA was then expressed in sex neurons using the $P_{unc\text{-}103F}$ promoter and in sex muscles using the $P_{unc\text{-}103E}$ promoter [63]. I previously showed that expressing wild-type *unc-103* genomic DNA in the sex muscles was sufficient to rescue

unc-103(0)-induced spicule protraction (See Chapter III) [49]. However, adding either *sy557* mutation interferes with the ability of sex-muscle *unc-103* to rescue spicule protraction (Table C-1). This indicates that both mutations affect channel function in a manner that makes it difficult to inhibit spicule protraction. On the other hand, adding *unc-103* containing either *sy557* point mutation has no effect on spicule protraction, similar to adding wild-type *unc-103* genomic DNA to neurons (Table C-1) [49]. While adding *unc-103* genomic DNA containing either *sy557* point mutation does not inhibit precocious spicule protraction, it does not increase the instance of protraction to levels seen in *unc-103(sy557)* males, either. This indicates that expressing *unc-103* containing either mutation in the sex muscles is insufficient to induce high levels of protraction. There are two possibilities: either both point mutations expressed on *unc-103* in the sex muscles are necessary to interfere with non-*unc-103*-mediated spicule protraction regulation, or one point mutation expressed in more than one area is necessary. To test these possibilities, I expressed *unc-103* carrying both *sy557A* and *sy557B* in the sex muscles of *unc-103(0)* and wild-type males and also *sy557B* in both the sex muscles and neurons of *unc-103(0)* males. Since the *unc-103(sy557)* allele is dominant, I asked if expressing *unc-103* carrying both mutations in the sex muscles or neurons induces protraction in wild-type males. I found that sex muscle or neuronal *unc-103(sy557A+B)* is unable to induce protraction, indicating that *unc-103(sy557)* needs to be in more than one tissue type to inhibit spicule protraction (Table C-1). Similarly, I found that sex-muscle *unc-103(sy557A+B)* had no affect on *unc-103(0)*-induced spicule protraction (Table C-1). In contrast, *unc-103(sy557B)* expressed in both sex muscles and neurons

increases spicule protraction to 62%, significantly higher than *unc-103(0)*-induced spicule protraction and similar to *unc-103(sy557)*-induced protraction (Table C-1). Thus, the *unc-103(sy557B)* point mutation is causing abnormal spicule protraction by interfering with cell excitability in both muscle and neurons. While the *unc-103(sy557A)* mutation does interfere with channel function, as sex-muscle *unc-103(sy557A)* is unable to rescue *unc-103(0)*-induced protraction, the *unc-103(sy557B)* mutation results in the dominant-negative aspects of UNC-103(*sy557*) channel function. However, what aspect of cell excitability UNC-103(*sy557*) is interfering with in both sex muscles and neurons has yet to be determined.

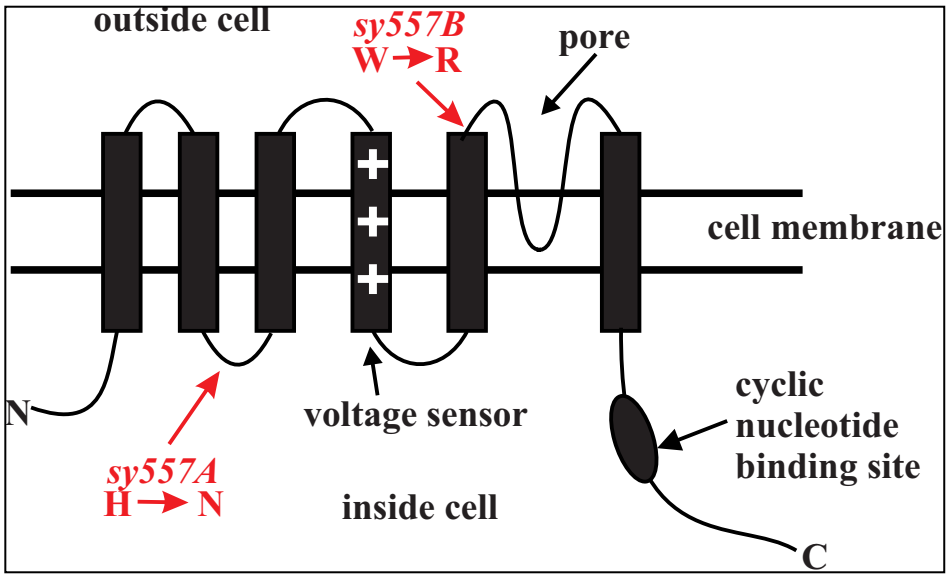

Figure C-1. Location of *sy557* mutations in the ERG-like K+ channel UNC-103. Diagram of UNC-103 structure. Black rectangles indicate transmembrane domains. The voltage-sensing transmembrane domain is indicated by the "+" symbol. Lines between rectangles indicate linker regions. The location of the *sy557* mutations is indicated in red. The potential cyclic nucleotide binding site is indicated by a black oval. N = N-terminal domain, C = C-terminal domain.

Table C-1. Effect of *sy557* mutations on spicule protraction		
Relevant Genotype[a,b]	**% Protraction (*n*)**	***p* value**[c]
unc-103(sy557)	75 (40)	
unc-103(0)	26 (53)	
unc-103(0); sex muscle sy557A	22 (45)	
unc-103(0); sex neuron sy557A	25 (40)	
unc-103(0); sex muscle sy557B	9 (33)	0.0567 to *unc-103(0)*
unc-103(0); sex neuron sy557B	23 (40)	
unc-103(0); sex muscle + sex neuron sy557B	62 (52)	0.0004 to *unc-103(0)*
Wild-type *sex muscle sy557A+B*	0 (51)	
unc-103(0); sex muscle sy557A+B	11 (35)	0.1097 to *unc-103(0)*
Wild-type *sex neuron sy557A+B*	0 (37)	
slo-1(lf) egl-2(0) control for *sex muscle sy557B*[d]	33 (55)	
slo-1(lf) egl-2(0); sex muscle sy557B	72 (89)	<0.0001 to control
slo-1(lf) egl-2(0) control for *sex neuron sy557B*	39 (31)	
slo-1(lf) egl-2(0); sex neuron sy557B	32 (47)	0.6288 to control
slo-1(lf) egl-2(0) control for *sex muscle + sex neuron sy557B*	36 (83)	
slo-1(lf) egl-2(0); sex muscle + sex neuron sy557B	81 (32)	<0.0001 to control
unc-103(0); slo-1(lf) egl-2(0)	79 (42)	

[a]All strains contain *him-5(1490)*

[b]All strains except *unc-103(sy557)* contain *pha-1(e2123)*

[c]Fisher's Exact Test

[d]*slo-1(lf) egl-2(0)* controls are siblings of males expressing the transgene

***unc-103(sy557B)* expressed in sex muscles induces protraction in *slo-1(lf) egl-2(0)* males**

In addition to the effects of *unc-103(sy557)* on wild-type and *unc-103(0)* males, I also looked at *unc-103(sy557B)* interference with *slo-1* and *egl-2* K+ channels. All three K+ channels are involved in reducing male sex-muscle excitability, and removing the function of all three results in a 79% rate of spicule protraction (n = 42) (Table C-1). While *egl-2* appears to only be active under food deprivation conditions, both *slo-1* and *unc-103* are necessary to inhibit premature contractions when food is present (see Chapters III and V) [12,49]. However, they appear to work together in some manner, as an *unc-103(0); slo-1(lf)* double mutant does not show an additive affect of the two single mutants (see Chapter V). To further explore a possible interaction between the two K+ channels, I expressed *unc-103(sy557B)* in the sex muscles and neurons of *slo-1(lf) egl-2(0)* males. I found that expressing *unc-103(sy557B)* in the sex neurons has no affect on spicule protraction, but expressing *unc-103(sy557B)* in the sex muscles or in both the sex muscles and neurons increases the rate of protraction from to 72% and 81%, respectively (Table C-1). This indicates that sex-muscle *unc-103(sy557B)* interferes with mechanisms able to compensate for the loss of *slo-1* function. In addition, the triple mutant *unc-103(0); slo-1(lf) egl-2(0)* displays a similar rate of spicule protraction, indicating that sex-muscle expression of *unc-103(sy557B)* is acting in a dominant-negative fashion to disrupt *unc-103* channel function. In *unc-103(0)* males, *unc-103(sy557B)* has to be expressed in sex muscles and neurons to induce *unc-103(sy557)*

levels of spicule protraction. In contrast, *unc-103(sy557B)* only needs to be expressed in the sex muscles of *slo-1(lf) egl-2(0)* males to greatly increase spicule protraction, and expressing it additionally in the sex neurons does not increase the instance of the mutant phenotype further. Therefore, *slo-1* and/or *egl-2* can act as a buffer for *unc-103(sy557B)*-induced protraction in the sex muscles, and needs properly functioning neurons to do so. I favor a model in which *slo-1* is acting in the sex muscles to compensate for disrupted *unc-103* function due to the fact that *slo-1* is active under well-fed conditions and *egl-2* is not.

APPENDIX D

PCA AND PCB NEURONS REGULATE MALE POSITIONING AT THE VULVA

PCA and PCB neurons

I examined the role of the post cloaca sensilla (p.c.s.) PCA and PCB neurons in regulating male mating behavior. The PCA and PCB consist of left/right pairs, their neuronal bodies are located in the male tail, and they send processes just posterior to the cloaca opening [5]. They are connected to the spicule protractor muscles and the PCB connects to the anal depressor muscle via gap junctions; the protractors induce spicule protraction while the anal depressor has no know function (Figure D-1) (Male Wiring Project, http://worms.aecom.yu.edu/pages/male_wiring_project.htm) [29]. In addition, they synapse the hook sensillum that send processes just anterior to the cloaca opening and PCB synapses the SPC motor neurons that control spicule insertion (Figure D-1) [29]. Both the p.c.s. neurons and hook sensillum are involved in the vulva location step of male mating behavior, as removing these neurons from males interferes with their ability to maintain position at the vulva and locate the vulva, respectively [29]. It has also been reported that the hook sensillum and p.c.s. neurons induce the spicule prodding behavior necessary for the spicules to penetrate the tightly closed vulva [47]. Katharine Liu reported that while ablating any two of the three pairs of p.c.s. neurons (PCC is the third neuron pair) interfered with a male's ability to maintain position at the vulva, ablating the neurons individually resulted in no observed defect [29]. However, she did

not closely monitor how the males behaved at the vulva and it is unlikely that in an animal with so few neurons three pairs would have identical overlapping functions. I looked at the effect of individually removing the PCA and PCB neurons on the male's ability to maintain their position at the vulva.

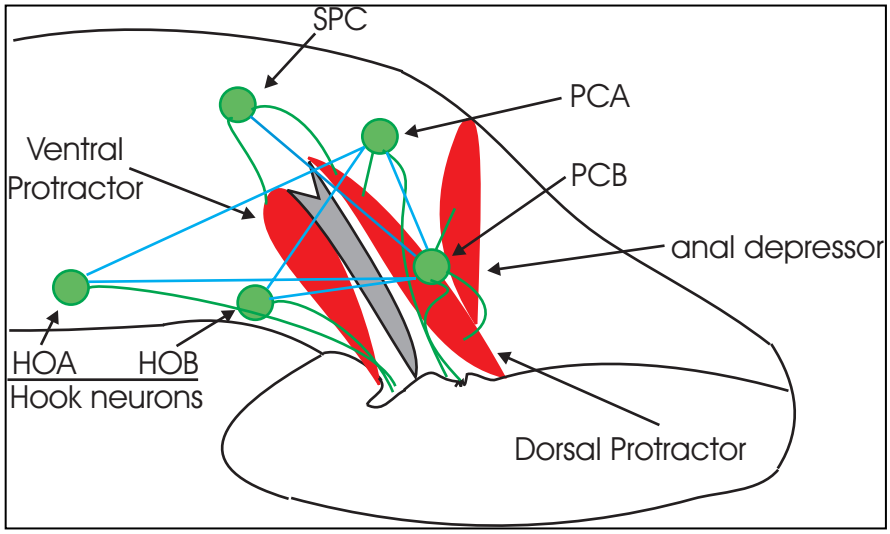

Figure D-1. Connectivity between neurons and muscles in the male tail. Diagram of male tail. Neurons are indicated by green, muscles by red, and the spicule is gray. Connectivity between neurons and muscles is indicated in green. Connectivity between neurons is indicated in blue.

To determine the individual contributions of PCA and PCB to vulva location, I examined the ability of males lacking these neurons to maintain vulva position. I ablated the left/right pairs of either PCA or PCB from wild-type males at the L4 stage, allowed the males to mature to adults overnight, and assayed their ability to stay at the vulva of paralyzed hermaphrodites. The ability of males to maintain their position at the vulva

was analyzed in three ways: average time spent at the vulva per stop, percentage of time spent at the vulva, and the number of times a male left the vulva. The first two tests looked at how long a male is spending at the vulva. To mate successfully, a male needs to stay at the vulva until spicule insertion is obtained, so sperm can be transferred. The less amount of time a male spends at the vulva, the less likely he is going to be successful in siring progeny. The third step looked at the number of times a male left the vulva while in contact with a hermaphrodite. In two tests, average time per vulva stop and percentage of time spent at the vulva compared to total time spent in contact with the hermaphrodite, both PCA and PCB ablated males underperformed their wild-type counterparts (Figure D-2A-B). Thus, both neuron pairs contribute to a male's ability to maintain position at the vulva, and removing even one impairs mating ability. In contrast, in the vulva leaving assay, males lacking PCB but not PCA leave the vulva more the wild-type, indicating that their interest in maintaining their position at the vulva is reduced (Figure D-2C). Thus, while both neurons are required to maintain vulva position, PCB plays an additional role in keeping the males at the vulva.

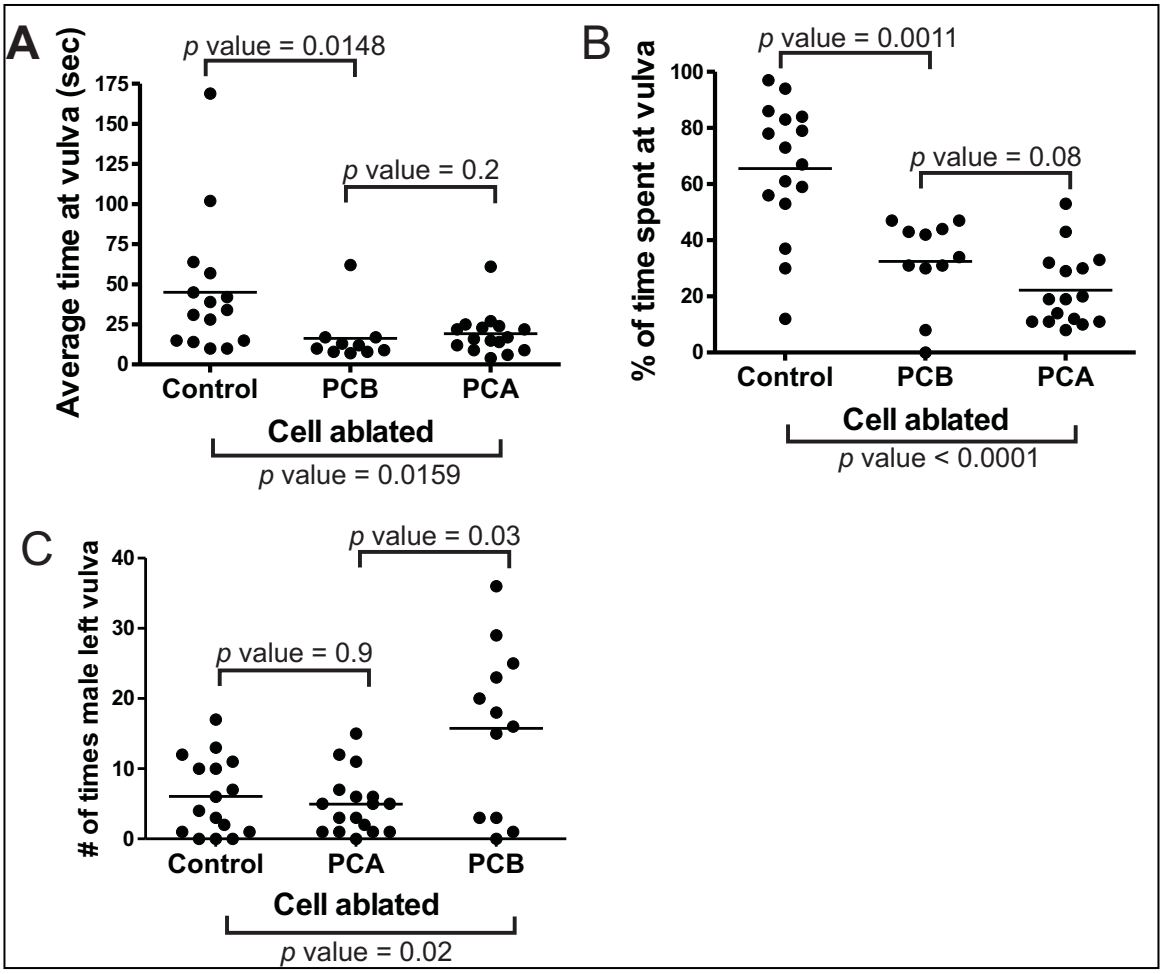

Figure D-2. Ablated male ability to maintain position at vulva. (A) Average time per stop at the vulva. The spots represent the average time one male spent at the vulva per stop. *x* axis indicates the cell ablated, the *y* axis is in seconds. (B) Percentage of time in contact with a hermaphrodite that is spent at the vulva. The spots represent one male each. *x* axis indicates the cell ablated, the *y* axis indicates the percentage of time spent at the vulva. (C) Vulva leaving assay. The spots represent the number of times an individual male left the vulva. *x* axis indicates the cell ablated, the *y* axis indicates the number of times a male left the vulva. For all graphs, Control $n = 16$, PCA $n = 17$, PCB $n = 12$. The *p* value was calculated using the Mann-Whitney test.

APPENDIX E

INTERACTIONS BETWEEN MUSCLE REGULATORY PROTEINS AND CaMKII/UNC-43

A tropomyosin mutation that inhibits *unc-103(lf)*-induced spicule protraction does not affect *unc-43(sy574)*-induced spicule protraction under standard conditions

Previously, I identified CaMKII/*unc-43* and ERG-like K+ channel/*unc-103* function in the muscle as necessary to suppress premature spicule protraction (see Chapter III) [49]. In addition, *unc-43* works parallel to *unc-103* in suppressing premature sex muscle contraction. To determine the role of *unc-43* in the muscle in comparison to *unc-103*, I looked at the interaction of the *unc-43* mutant *sy574* with a loss-of-function in tropomyosin/*lev-11*. Tropomyosin is a muscle regulatory protein that runs along the length of the actin fiber and prevents actin-myosin interaction, thereby inhibiting muscle contraction [10,140,141]. A loss-of-function mutation in *lev-11*, *rg1*, was identified in a screen looking for suppressors of *unc-103(sy557)*-induced spicule protraction [28]. Since *lev-11(rg1)* suppresses *unc-103(sy557)*-induced spicule protraction, I hypothesized that it would not affect protraction in *unc-43(sy574)* males. I made a *lev-11(rg1); unc-43(sy574)* double mutant and found that *lev-11(rg1)* as no effect on *unc-43(sy574)*-induced spicule protraction (Table E-1). Thus, *unc-43* and *unc-103* are functioning in separate pathways to suppress sex muscle excitability, and those pathways are separate in the way they regulate muscle regulatory proteins.

Table E-1. Tropomyosin/*lev-11* mutant interaction with *unc-43(sy574)*		
Genotype[a]	**% Protracted (*n*)**	***p* Value**[b]
Wild-type	12 (106)	
tropomyosin/*lev-11(rg1)*	0 (26)	
ERG/*unc-103(sy557)*	82 (43)	<0.0001 to wt
lev-11(rg1); unc-103(sy557)	17 (126)	<0.0001 to *unc-103(sy557)*
CaMKII/*unc-43(sy574)*	48 (79)	
lev-11(rg1); unc-43(sy574)	56 (54)	

[a]Strains contain *him-5(e1490)*

[b]Fisher's Exact Test

A mutation in tropomyosin affects *unc-43(sy574)* differently under varying temperatures

Temperature differences can affect protein folding and, as a consequence, mutant phenotypes. For example, animals carrying the temperature sensitive allele *pha-1(e2123ts)* appear normal at 15°C, but are unable to survive at 20°C, because the *pha-1(e2123ts)* protein created at this temperature is unable to perform its role in pharyngeal development [54]. The tropomyosin/*lev-11(rg1)* allele also appears to display temperature-sensitive effects. This allele causes a point mutation near a troponin T (TNT) binding site in tropomyosin; as TNT is a regulatory protein, mutating this site presumably interferes with tropomyosin regulation [11,28,66]. At 20°C, *lev-11(rg1)* is able to suppress *unc-103(0)*- but not *unc-43(sy574)*-induced spicule protraction. I looked at the ability of *lev-11(rg1)* to inhibit the *unc-43(sy574)* phenotype at 15°C and

25°C. Interestingly, *lev-11(rg1)* inhibits *unc-43(sy574)*-induced protraction at 25°C and exacerbates the *unc-43(sy574)* phenotype at 15°C, while changing temperatures has the opposite effect on the *unc-43(sy574)* allele alone (Table E-2, Figure E-1). This suggests that the *lev-11(rg1)* allele is able to interfere with *unc-43* control of muscle excitability under different conditions. Under standard conditions, the regulatory protein binding site mutated in the *lev-11(rg1)* allele plays no role in *unc-43*-mediated regulation of muscle contraction. However, under stressful conditions such as high or low temperatures, this regulatory site becomes important in the *unc-43* pathway that mediates muscle excitability. *lev-11(rg1)* is able to suppress both *unc-43(sy574)*- and *unc-103(sy557)*-induced spicule protraction phenotype at 25°C (Table E-2). Therefore, while this regulatory site in tropomyosin is only important for *unc-103*-mediated sex muscle excitability at 20°C, it plays a role for both *unc-103* and *unc-43* at different temperatures.

Table E-2. Temperature Affects Penetrance of *lev-11(rg1); unc-43(sy574)* Phenotype

	Temperature (C)		
Genotype[a]	15 (*n*)	20 (*n*)	25 (*n*)
Wild-type	3% (36)	0% (40)	8% (48)
unc-43(sy574)	43% (51)	48% (79)	60% (55)
lev-11(rg1); unc-43(sy574)	73% (48)	56% (54)	16% (95)
lev-11(rg1); unc-103(sy557)	N.D.[b]	18% (76)	15% (27)

[a]Strains contain *him-5(e1490)*

[b]Not Done

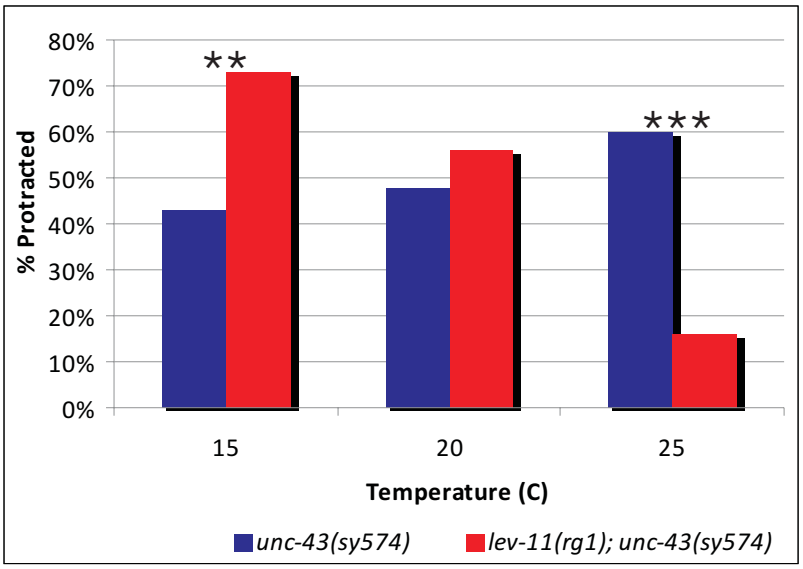

Figure E-1. Temperature effects on *lev-11(rg1); unc-43(sy574)*. Graph indicates the percentage of males that protracted their spicules at a given temperature. *x* axis is the temperature (C), *y* axis is the percentage of males protracting their spicules. ** *p* value < 0.005, Fisher's Exact Test. *** *p* value < 0.0005, Fisher's Exact Test. *n* values are the same as in Table E-2.

Muscle regulatory protein troponin T is involved in CaMKII-mediated spicule protraction

Tropomyosin is regulated by the three-subunit troponin complex. Troponin T binds troponins I and C to regulate muscle excitability [11]. Even the tropomyosin/*lev-11(rg1)* allele does not affect *unc-43(sy574)*-induced spicule protraction at 20°C, tropomyosin and its associated troponin complexes are ubiquitous muscle regulatory proteins that are likely downstream of *unc-43*'s regulation of spicule protraction. *C. elegans* contains four different troponin T genes. Todd Gruninger identified the pharyngeal-expressed *tnt-4* as involved in *unc-103*-mediated suppression of sex muscle excitability [28]. Since *unc-103* appears to regulate male sex muscle excitability

separately from *unc-43*, it is likely that they have different TNT proteins as downstream targets. I performed RNA interference (RNAi) experiments to knockdown the function of the different TNT genes in *unc-43(sy574)* males. *unc-43(sy574)* males were selected as L4s and allowed to develop overnight in liquid containing RNAi targeted to the different TNT genes. I found that reducing the amount of *tnt-1* and *tnt-3* affect *unc-43(sy574)*-induced spicule protraction (Table E-3) [28]. Thus, in contrast to *unc-103*, *unc-43* affects muscle regulation through *tnt-1* and *tnt-3*.

Table E-3. Affect of TNT RNAi on *unc-43(sy574)* Phenotype

RNAi	% Protraction	*n*	*p* value[a]
none	59%	27	
tnt-1	20%	20	0.0089
tnt-2	37%	30	0.1144
tnt-3	17%	36	0.0011
tnt-4	39%	18	0.2307

[a]Fisher's Exact Test

APPENDIX F

MATING VARIABILITY BETWEEN *C. ELEGANS* STRAINS

N2, the *C. elegans* strain I used for my studies, does not display an especially impressive ability to sire progeny. When virgin one-day-old males are given 20 min with a paralyzed hermaphrodite, 50% are able to sire at least one offspring (n = 30). While watching mating behavior, I and others have noticed that some males immediately perform the mating steps while others never seem interested in the hermaphrodite. In an attempt to analyze if there is inherent variability in N2 mating, I looked at the ability of individual males to mate repeatedly over a period of two days. Individual males were isolated as L4s and allowed to develop into adults overnight. In the morning, each virgin one-day-old male was mated with a two-day-old paralyzed *unc-64(lf)* hermaphrodite for 20 min, given 40 min to rest, and mated again with a different paralyzed hermaphrodite. This was repeated until each male had mated 5 times on the first day, and 5 times on the second day, for a total of 30 males. The males were given a 40 min rest period based on data that says males undergo a quiescent period of approximately 20 min after each successful mating before they are interested in mating again (Pinky Mehta and L. Rene Garcia, unpublished observation). 6 of the males either displayed permanent spicule protraction after a few mating attempts or committed suicide by crawling up the side of the plates and desiccating; these males were removed from the final numbers. I found there exists a great deal of variability from male to male in the N2 population (Figure F-1). Out of ten tries, males mated anywhere from 0 to 9 times, with the most males

mating 5 times. The virgin adult males chosen for this study all appeared morphologically and behaviorally normal, and the bacteria lawn on which mating occurred was uniform in size, so these factors are not contributing to the variability. Unseen morphological differences could account for the variability, as could differences at the cellular and molecular level.

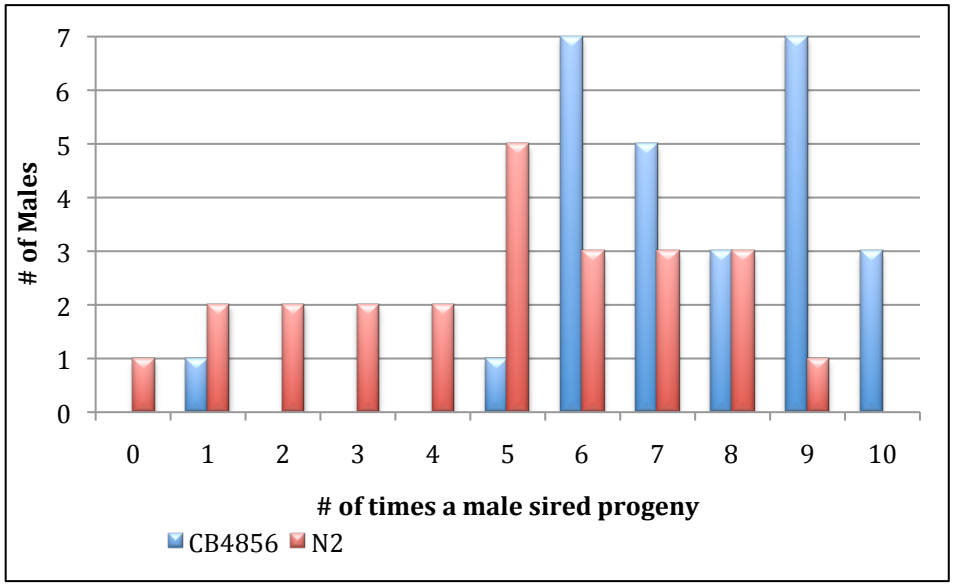

Figure F-1. Mating success. Number of males able to sire at least one progeny a certain number of times. *x*-axis is the number of times a male sired progeny, and the *y*-axis is the number of males that sired at least one progeny. $n = 27$ for CB4856, $n = 24$ for N2.

First isolating and then studying N2 males with improved mating behavior would be a difficult and time-consuming task, especially at the cellular and molecular level. Instead, I chose to look at another strain, CB4856, that has been reported to have increased mating success compared to N2 [134]. I performed the same mating assay as I

had with N2, where 30 CB4856 males were given 10 chances to sire progeny over a period of 2 days. 3 males committed suicide and were removed from the final analysis. I found that a greater percentage of matings occurred overall as compared to N2 (75% to 49%, Figure F-2), and variability was decreased, as most CB4856 males mated 6 to 10 times, with only 2 males mating less than 6 times (Figure F-1). In addition, for most individual attempts, CB4856 showed improved mating ability (Figure F-3). One difference between the N2 and CB4856 strains is that the N2 strain carried a mutation, *him-5(e1490)*, to increase the instance of males in the population, and CB4856 did not. However, I do not believe the *him-5(1490)* accounts for the decreased mating performance seen in N2 males. First off, *him-5* affects chromosomal nondisjunction, not behavior. Secondly, another lab that reported mating differences in N2 and CB4856 strains did not use the *him-5(1490)* mutation, and instead used wild-type N2 males [134]. The mating differences between the N2 and CB4856 strains provides an opportunity to analyze the molecular and cellular controls of mating.

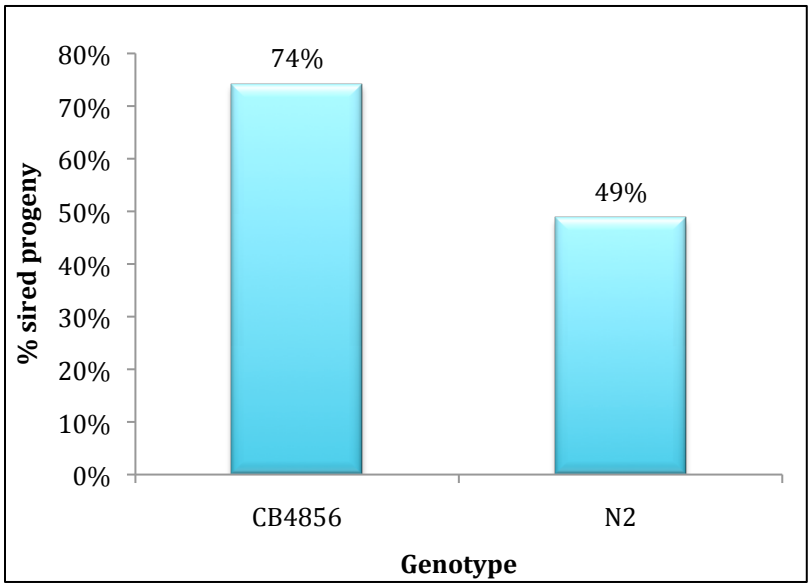

Figure F-2. Male potency. Percentage of males capable of siring at least one progeny for CB4856 or N2 males. *x*-axis is the genotype, and the *y*-axis is the percentage of males that sired at least one progeny. $n = 270$ for CB4856, $n = 240$ for N2. p value < 0.0001, Fisher's Exact Test.

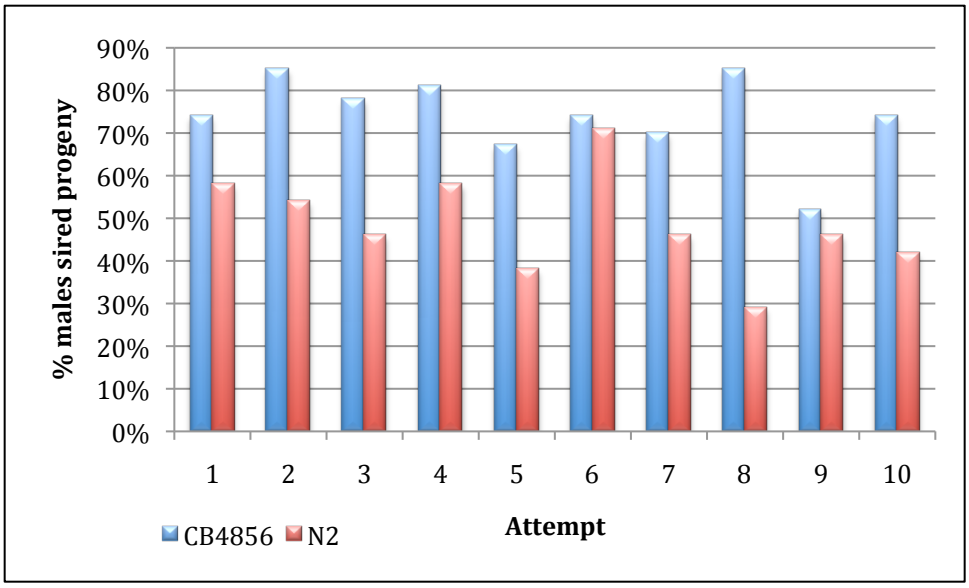

Figure F-3. Male potency per attempt. Percentage of males capable of siring at least one progeny per individual mating attempt. *x*-axis is the attempt, and the *y*-axis is the percentage of males that sired at least one progeny. $n = 27$ for CB4856, $n = 24$ for N2. Attempts 2, 3, 8, and 10 have p values < 0.05, Fisher's Exact Test.

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PEER-REVIEWED PUBLICATIONS

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