Food Deprivation Attenuates Seizures through CaMKII and EAG K^+ Channels

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Accumulated research has demonstrated the beneficial effects of dietary restriction on extending lifespan and increasing cellular stress resistance. However, reducing nutrient intake has also been shown to direct animal behaviors toward food acquisition. Under food-limiting conditions, behavioral changes suggest that neuronal and muscle activities in circuits that are not involved in nutrient acquisition are down-regulated. These dietary-regulated mechanisms, if understood better, might provide an approach to compensate for defects in molecules that regulate cell excitability. We previously reported that a neuromuscular circuit used in *Caenorhabditis elegans* male mating behavior is attenuated under food-limiting conditions. During periods between matings, sex-specific muscles that control movements of the male's copulatory spicules are kept inactive by UNC-103 *ether-a-go-go–related gene* (ERG)–like K^+ channels. Deletion of *unc-103* causes ~30%–40% of virgin males to display sex-muscle seizures; however, when food is deprived from males, the incidence of spontaneous muscle contractions drops to 9%–11%. In this work, we used genetics and pharmacology to address the mechanisms that act parallel with UNC-103 to suppress muscle seizures in males that lack ERG-like K^+ channel function. We identify calcium/calmodulin-dependent protein kinase II as a regulator that uses different mechanisms in food and nonfood conditions to compensate for reduced ERG-like K^+ channel activity. We found that in food-deprived conditions, calcium/calmodulin-dependent protein kinase II acts cell-autonomously with *ether-a-go-go* K^+ channels to inhibit spontaneous muscle contractions. Our work suggests that upregulating mechanisms used by food deprivation can suppress muscle seizures.

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Introduction

The excitability of neuromuscular circuits must be regulated to ensure appropriate behavioral responses under different conditions. When cell excitability is irregular due to channelopathy defects, inappropriate motor output can lead to different medical conditions. One member of the *ether-a-go-go* (EAG) family of K^+ channels, the human *ether-a-go-go–related gene (hERG)*–encoded, delayed inward, rectifying voltage-gated K^+ channel, has received attention due to its association with the cardiac condition long QT syndrome [1]. Most mutations in *hERG* reduce channel conductance, which causes prolonged depolarizations that result in cardiac arrhythmias [2–6]. Due to the physiological significance of this protein, there is ongoing research into the biophysical properties of hERG K^+ channel function. However, little is known about other signaling pathways that act with these channels to control excitable output within specific physiological contexts.

We reported previously that the *Caenorhabditis elegans* homolog of hERG, UNC-103, regulates the movements of the male's copulatory spicules before and during male mating behavior [7]. Prior to mating, the two spicules are held within the male tail via their attachments to dorsal and ventral protractor and retractor muscles. During mating, males rhythmically contract the spicule muscles to protract their spicules through the hermaphrodite vulva. Similar to the irregular cardiac muscle depolarizations caused by defective *hERG*, deletion of *unc-103* causes spontaneous contractions of the spicule muscles, inducing spicule protraction in the absence of mating cues. Interestingly, the *unc-103(0)* deletion phenotype is incompletely penetrant, suggesting that redundant pathways regulate this motor output [7,8].

The incidence of spontaneous spicule muscle seizures in *unc-103(0)* males is reduced by food deprivation [9]. This suggests that signal transduction pathways, activated under nutrient-poor conditions, specifically modulate the expression of the *unc-103(0)* phenotype. Food deprivation globally modulates many behaviors to direct the animal toward food acquisition [10–13]. In *C. elegans* and higher organisms, dietary deprivation not only represses non–food-foraging behaviors, but also has positive physiological effects on stress resistance and aging. This suggests mechanisms, activated under food-deprived conditions, are possible targets to treat illnesses caused by channelopathies and stress. Food deprivation likely modulates diverse behavioral outputs by acting on different

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Abbreviations: ARE, arecoline; CaMKII, calcium/calmodulin-dependent protein kinase II; *lf*, loss-of-function; CFP, cyan fluorescent protein; EAG, *ether-a-go-go*; ERG, *ether-a-go-go–related gene*; L-VGCC, L-type voltage-gated Ca^{++} channel; NGM, nematode growth medium; NSM, neurosecretory motor; RyR, ryanodine receptor Ca^{++} channel; YFP, yellow fluorescent protein

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Author Summary

We investigated the mechanisms used during dietary stress conditions that regulate the behavioral output of excitable cells. In the roundworm *Caenorhabditis elegans,* males must display the proper behavioral response to potential food sources or mating partners. This regulation is disrupted by loss of ERG-like K^+ channel function. K^+ channel defects cause a percentage of males to contract their genitalia muscles permanently, even in the absence of mating cues. For the percentage of K^+ channel–defective males that do not display spontaneous sex-muscle seizure, we show that abnormal muscle contraction is attenuated by the calcium/calmodulin-dependent protein kinase II (CaMKII). Under food-deprived conditions, we find that CaMKII acts with the *ether-a-go-go* K^+ channel to further suppress spontaneous sex-muscle seizures. We speculate that in food-abundant conditions, *ether-a-go-go–related gene*–like K^+ channels act in parallel with a CaMKII-regulated pathway(s) to attenuate sexual behavior until mating signals are presented. If one of these mechanisms is disrupted, the other can compensate. In the absence of food, CaMKII and *ether-a-go-go* K^+ channels further hyperpolarize the genital neuromuscular circuitry, changing the behavioral state so that the male will forage for food rather than mate.

molecular regulators within specific neuromuscular circuits. We previously reported that feeding behavior influences the spicule circuit via signaling from the muscular feeding organ, the pharynx, and pharyngeal-associated neurons [9]. In this study, we used the feeding state/spicule protraction relationship in *C. elegans* to identify a key molecular regulator involved in suppressing defective ERG-like K^+ channel/UNC-103 muscle contractions.

We found that calcium/calmodulin-dependent kinase II (CaMKII) is a central regulator within the spicule circuit that modulates sex-muscle excitability under both well-fed and food-deprived conditions. Biochemical and genetic studies in *C. elegans* and *Drosophila* have independently linked CaMKII activity with the K^+ channel function of ERG and its close relative EAG [14–16]. In this study, we describe two physiological contexts in which UNC-43, the worm homolog of CaMKII, works with ERG-like and EAG K^+ channels to regulate a specific muscle output. Specifically, we propose that under well-fed conditions, CaMKII/UNC-43 works redundantly with ERG-like K^+ channel/UNC-103 to suppress spicule protraction in periods between mating, while under food-deprived conditions, CaMKII/UNC-43 and EAG K^+ channels suppress spicule protraction via a parallel mechanism.

Results

sy574 Defines a Pathway Separate from ERG-like K^+ channel/*unc-103(0)*

Previously, we found ERG-like K^+ channel/UNC-103 suppresses spontaneous sex-muscle contraction prior to mating [7]. *C. elegans* are normally grown on nematode growth medium (NGM) plates containing a lawn of OP50 bacteria. Under these conditions, 42% of *unc-103(0)* males display constitutive spicule protraction (Table 1). Since deletion of *unc-103* causes less than half of the males in a population to protract their spicules permanently, we hypothesized that additional mechanisms regulating sex-muscle contraction are functioning in the non–spicule-protracted males. To determine these additional mechanisms, we analyzed a mutant allele, *sy574*, that was isolated in the same screen that identified *unc-103* as a regulator of spicule protraction [7]. *sy574* induces spicule protraction in 56% of males (Table 1); hermaphrodites move, lay eggs, defecate, and respond to mechanosensation normally (unpublished data), demonstrating that *sy574* disrupts one behavior.

Table 1. Abnormal Spicule Protraction Induced by Mutant CaMKII/*unc-43* and ERG-Like K^+ Channel/*unc-103* Alleles

Genotype[a]	Percent Spicule-Protracted Males (*n*)	*p* Value[b]
Wild-type	12 (106)	
ERG-like K^+ channel/*unc-103(0)*	42 (91)	<.005 to wt
CaMKII/*unc-43(sy574)*	56 (300)	<.005 to wt
unc-103(0); unc-43(sy574)	97 (92)	<.005 to wt
RyR/*unc-68(0)*	15 (26)	
L-VGCC/*egl-19(lf)*	2 (41)	
unc-43(sy574); unc-68(0)	46 (37)	
unc-103(0); unc-68(0)	8 (48)	
egl-19(lf); unc-43(sy574)	0 (48)	<.005 to *unc-43(sy574)*
EAG K^+ channel/*egl-2(gf)*	0 (30)	
egl-2(0)	11 (55)	
unc-43(sy574); egl-2(n693gf)	4 (57)	<.005 to *unc-43(sy574)*
unc-103(0); egl-2(n693gf)	43 (94)	0.91 to *unc-103(0)*
unc-43(n1186)	100 (58)	<.005 to wt
unc-43(n1186); egl-2(n693gf)	76 (50)	<.005 to *unc-43(n1186)*

[a]Strains contain *him-5(e1490)*.
[b]Fischer exact test.
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sy574 and ERG-like K^+ channel/*unc-103(0)* superficially induce the same phenotype and might affect the same regulatory pathway. In *sy574* and *unc-103(0)* males, we measured the timing of constitutive spicule protraction to determine the extent of the similarities between the mutant phenotypes. Males were allowed to develop into adults, and then checked every hour for the mutant phenotype. We found that after 5 h, 30% of *unc-103(0)* males had protruding spicules. In contrast, only 25% of *sy574* males (out of 56% that eventually display the mutant phenotype) displayed spontaneous spicule protraction. Thus, the majority of *sy574* males have sex-muscle seizures later than *unc-103(0)* males, suggesting these two mutations affect spicule protraction differently. To determine if *sy574* worked in a different pathway from *unc-103,* we constructed a *sy574; unc-103(0)* double mutant. A total of 97% of *unc-103(0); sy574* males displayed spontaneous spicule protraction, which is higher than the 75% expected if the two mutations acted additively (Table 1). This suggests that *sy574* disrupts a separate signaling pathway from *unc-103(0).*

sy574 Is an Allele of CaMKII/*unc-43*

We used single nucleotide polymorphism mapping to position the *sy574* lesion to a 570-kb region on Chromosome IV between cosmids R102 and K08F4 [17]. We then injected PCR products of candidate genes into *sy574* animals to test for rescue, and we also performed complementation tests with alleles of genes located in the region. Our complementation tests suggested that the recessive *sy574* allele affects the

Table 2. Effect of Mutant CaMKII/*unc-43* Alleles on Male Spicule Protraction

Genotype[a]	Percent Spicule-Protracted Males (*n*)	*p* Value[b]
Wild-type	12 (106)[c]	
unc-43(sy574)	56 (300)[c]	
unc-43(sy574)/+	6 (79)	<.005 to *unc-43(sy574)*
unc-43(n1186)	100 (58)[c]	
unc-43(n1186)/+	6 (97)	<.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)	<.005 to *unc-43(sy574)*
unc-43(sy574)/unc-43(e408)	17 (72)	<.005 to *unc-43(sy574)*
unc-43(n498gf)	0 (32)	

[a]Strains contain *him-5(e1490).*
[b]Fischer exact test.
[c]Taken from Table 1.
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gene *unc-43,* since four loss-of-function *(lf) unc-43* alleles did not complement the *sy574* phenotype (Table 2). The non-complementing *unc-43(lf)* alleles, including the nonsense *unc-43(n1186)* allele, induce general locomotor defects, muscle seizures, and constitutive spicule protraction. Since *sy574* animals only displayed one of many *unc-43(lf)* phenotypes, we reason that *sy574* is a weak *lf* allele that encodes a protein with sufficient function to regulate muscles and neurons involved in general *C. elegans* behaviors. However, the spicule protraction circuit must be more sensitive than other cells to perturbations in UNC-43 function. The one allele that complemented *sy574* is *unc-43(e408)* (Table 2). *unc-43(e408)* males display locomotor and muscle seizure defects similar to animals containing the nonsense *unc-43(n1186)* allele; however, *unc-43(e408)* males do not display spontaneous spicule protraction (Table 2). Although *unc-43(e408)* animals do not display this abnormality, *unc-43(e408)* does affect spicule protraction, and its analysis is described in later sections.

unc-43 encodes the one *C. elegans* copy of CaMKII, a serine/threonine kinase responsible for phosphorylating multiple substrates that control many cellular functions [16]. CaMKII contains three functional domains: a N-terminal catalytic region responsible for substrate recognition, binding, and phosphorylization; an autoinhibitory domain responsible for blocking the catalytic region and keeping the protein inactive in the absence of calcium; and a C-terminal self-association domain by which the enzyme forms complexes of six- and 12-member rings [18]. Sequencing *unc-43* from *sy574* animals revealed two point mutations located in different functional domains. One mutation changes a glycine to glutamate at amino acid 170, near the substrate recognition domain of the catalytic region (Figure 1A) [19,20]. The second *sy574* mutation is located in the self-association domain and changes an alanine to valine at amino acid 465 (Figure 1A).

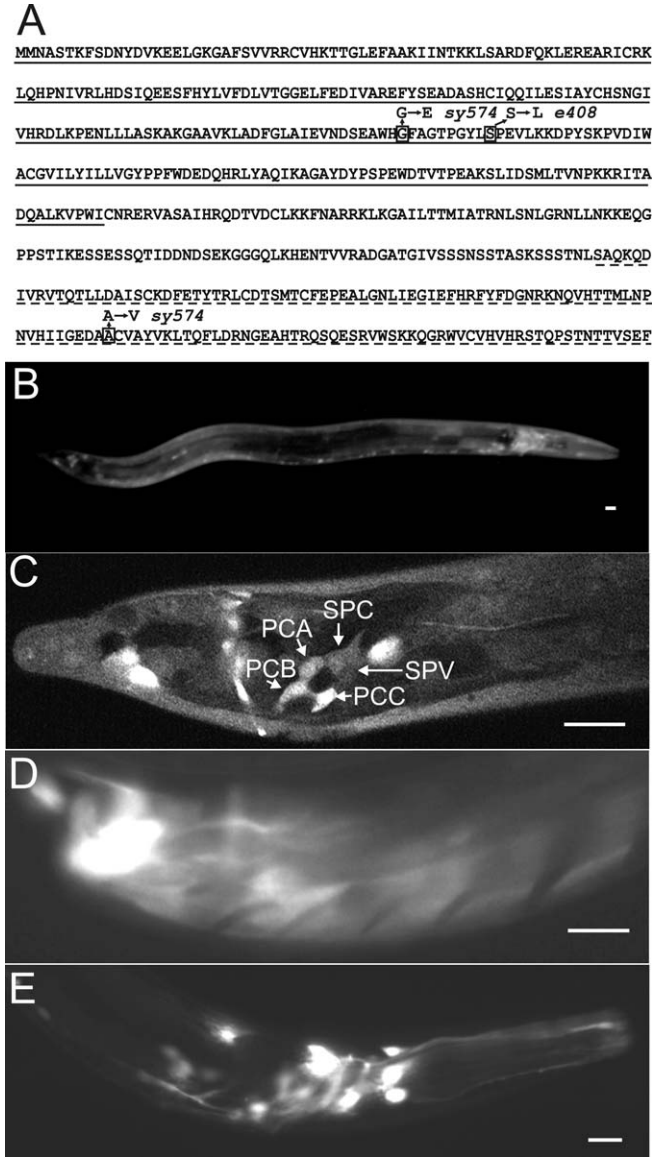

Figure 1. CaMKII/UNC-43 Amino Acid Changes Due to Mutations, CaMKII/*unc-43,* and EAG K^+ Channel/*egl-2* Expression Patterns

(A) Amino acid sequence of UNC-43 isoform g. The underlined region indicates the catalytic domain of CAMKII/UNC-43, the non-underlined region is the autoinhibitory domain, and the dashed underline indicates the self-association domain. *unc-43(sy574)* and *unc-43(e408)* point mutations are indicated by boxes. The amino acid change is listed next to the allele name.
(B) *unc-43* promoter:CFP expression in a L4 male.
(C) *unc-43* promoter:CFP expression in the L4 male tail. Arrows indicate neurons involved in male mating.
(D) *egl-2* promoter:YFP expression in an adult male tail.
(E) *egl-2* promoter:YFP expression in the adult male head.
In all figures, anterior is to the right, dorsal is to the top. Scale bar = 10 μM.
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CaMKII/UNC-43 and ERG-Like K^+ Channel/UNC-103 Act in Muscles to Regulate Spicule Protraction

Genetic analyses of *unc-43(sy574)* and *unc-103(0)* suggest that CaMKII/UNC-43 and ERG-like K^+ channel/UNC-103 act redundantly to suppress spontaneous sex-muscle contractions when food is available. Therefore, we asked which cells require their function under these standard conditions. The

Table 3. Transgenic Rescue of Mutant CaMKII/*unc-43* and ERG-like K^+ channel/*unc-103*–Induced Spicule Protraction

Genotype[a]	Tissue Expression	Percent Spicule-Protracted Males (*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)	.03
*unc-43(sy574);rgEx*164[P_{aex-3}::*unc-43(+)*]	Panneuronal	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(+)*]	Panmuscle	11 (98)	<.005
*unc-43(sy574); rgEx*120[P_{lev-11}::*unc-43–self-associated domain(+)*][c]	Panmuscle	15 (61)	<.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)	.06
unc-103(0)[c]		39 (67)	
*unc-103(0);rgEx*74[P_{aex-3}::*unc-103F(+)*][c]	Panneuronal	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]	Panmuscle	3 (30)	<.005
*unc-103(0);rgEx*79[$P_{unc-103E}$::*unc-103F (+)*][c]	Spicule protractor muscles + few head neurons	2 (44)	<.005
*unc-103(0);rgEx*81[$P_{unc-103E}$::*unc-103E (+)*][c]	Spicule protractor muscles + few head neurons	2 (46)	<.005

[a]Strains contain *him-5(e1490).*
[b]Fischer exact test; comparisons made to nontransgenic controls.
[c]Strain contains *pha-1(ts).*
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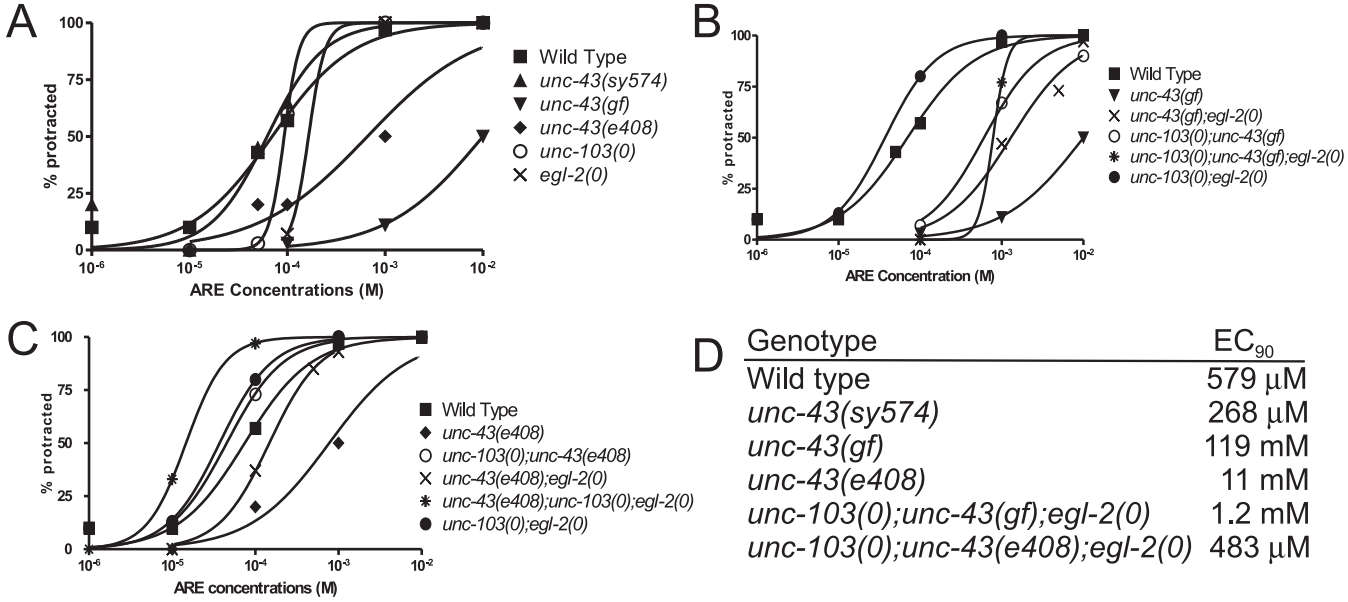
C. elegans male contains two spicules, each associated with two protractor and two retractor muscles and a nonessential accessory muscle (the anal depressor). Contraction of the protractor muscles forces the spicules through the cloacal opening, whereas contraction of the retractor muscles draws the spicules back into the tail. The SPC, hook, and the postcloacal sensilla neurons are functionally associated with the spicule muscles and trigger contractions upon mating stimulation [8,21,22]. Work by others using antibodies to rat CaMKII showed that in hermaphrodites, CaMKII/UNC-43 is broadly expressed in neurons, muscles, and intestines [16]. To determine if *unc-43* is expressed in the male spicule circuit, we PCR-amplified an 11-kb region upstream of the *unc-43* start codon, ligated the PCR fragment to the cyan fluorescent protein (CFP) gene, and injected the ligation product into *C. elegans*. In males, we found that CaMKII is broadly expressed in excitable cells, including the spicule protractor and retractor muscles, SPC, and postcloacal sensilla neurons (Figure 1B and 1C). For *unc-103,* we previously reported the *unc-103* loci are expressed from at least six promoters (promoters $P_{unc-103A}$ through $P_{unc-103F}$). These promoters express mRNA with distinct first exons that give rise to the isoforms *unc-103A* through *unc-103F*. Many of the promoters express *unc-103* broadly in neurons and muscles in both sexes. However, promoter $P_{unc-103E}$ expresses *unc-103* in approximately seven neuron pairs in the head, two pharyngeal neurons, and the spicule protractor muscles, but not spicule retractors in the tail, whereas $P_{unc-103F}$ expresses *unc-103* in multiple neurons, including the SPC and postcloacal sensilla neurons that control spicule muscle contractions [23].

To determine where CaMKII/*unc-43* is functioning in the male to suppress premature sex-muscle contraction, we created an *unc-43* cDNA construct using the full-length isoform *unc-43*g [24,25]. To determine if our construct was functional, we drove *unc-43* cDNA expression using the ubiquitously expressed *hsp-16* heat-shock promoter. We injected this construct into *unc-43(sy574)* animals and found that heat-shocked males showed significant reduction in *sy574*-induced spicule protraction compared to control siblings (Table 3). Since CaMKII/*unc-43* is expressed in many tissues, we wanted to identify where *unc-43* function is required to control spicule protraction. Using the *aex-3* promoter, which is broadly expressed in neurons, and the *tnt-4* promoter, which is expressed in the pharynx, we determined that *unc-43* expressed in these cell types has no effect on *unc-43(sy574)*–induced spicule protraction (Table 3) [9,26]. However, expressing *unc-43* via the *lev-11* pan–body-wall and sex-muscle promoter reduced the mutant phenotype (Table 3) [27]. Thus, CaMKII/*unc-43* is required in the muscles to prevent premature muscle contraction.

Once we had identified that muscle CaMKII/*unc-43* regulates spicule protraction, we asked which of the two point mutations in *unc-43(sy574)* affects normal kinase function. To address this, we generated an *unc-43* isoform lacking the self-association domain, but containing the kinase and inhibitory domains. We expressed this construct in muscles using the *lev-11* promoter, and found that it also rescued *unc-43(sy574)*–induced protraction (Table 3). This suggests that the *sy574* mutation in the catalytic domain, and not in the self-association domain of *unc-43,* is responsible for constitutive spicule protraction.

Since CaMKII/*unc-43* appears to regulate sex-muscle output via a separate mechanism than ERG-like K^+ channel/*unc-103*, we asked if *unc-103* also acts in muscles. The *unc-103* gene in *C. elegans* contains six first exons that produce tissue-specific isoforms. In hermaphrodites, the *unc-103*E isoform expressed from $P_{unc-103E}$ in sex muscles is required to rescue an *unc-103(0)*–induced egg-laying defect [23]. Therefore, in addition to determining which tissues require functional *unc-103* to control spicule protraction, we also asked if the rescue was isoform specific. We used isoform-specific *unc-103* genomic constructs, and found that the neuron-specific isoform *unc-103F,* expressed via the *aex-3* panneuronal promoter or the neuronal-specific $P_{unc-103F}$ promoter, did not rescue *unc-*



D

Genotype	EC_{90}
Wild type	579 μM
unc-43(sy574)	268 μM
unc-43(gf)	119 mM
unc-43(e408)	11 mM
unc-103(0);unc-43(gf);egl-2(0)	1.2 mM
unc-103(0);unc-43(e408);egl-2(0)	483 μM

Figure 2. ARE Pharmacology of *unc-43* Mutants

(A–C) Graphs of male muscle ARE sensitivity. The ARE concentrations are indicated on the *x*-axis, and the percent of males protracting their spicules in the drug are indicated on the *y*-axis. For each data point, $n = 30$ males.

(D) EC_{90} values at which 90% of males protracted their spicules for the genotypes listed.

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103(0)–induced protraction (Table 3). However, the *unc-103F* and *unc-103E* isoforms driven in muscles via the *lev-11* panmuscle promoter or by the sex-muscle promoter $P_{unc\text{-}103E}$ restored control of spicule protraction in *unc-103(0)* males (Table 3). Thus, similar to CaMKII/*unc-43*, ERG-like K^+ channel/*unc-103* acts in muscles to regulate spicule protraction, and unlike hermaphrodite egg-laying behavior, specific *unc-103* isoforms are not essential for regulation.

Interestingly, although both CaMKII/UNC-43 and ERG-like K^+ channel/UNC-103 act in muscles, the functional expression of *unc-43* in regulating spicule muscle contraction is not as restricted as *unc-103*. *unc-43*, when expressed broadly in body-wall muscles and all sex muscles from the *lev-11* promoter, rescued the *unc-43(sy574)*–induced spicule protraction. However, *unc-43* expressed in body-wall muscles from the *acr-8* promoter (see Text S1) or expressed in the sex muscles from the $P_{unc\text{-}103E}$ promoter was not sufficient to suppress constitutive spicule protraction (Table 3). This suggests that under standard conditions, UNC-43 might act in both body-wall and spicule protractor muscles to regulate spicule protraction behavior. Alternatively, since *lev-11* but not the $P_{unc\text{-}103E}$ promoter drives transcription in the retractor muscles, UNC-43 might be required in the protractor and retractor muscles, whereas UNC-103 is required only in the protractor muscles.

CaMKII/*unc-43* Requires EAG K^+ Channel/*egl-2* and ERG-Like K^+ Channel/*unc-103* to Regulate Sex-Muscle Excitability

Since both CaMKII/*unc-43* and ERG-like K^+ channel/*unc-103* are functioning in muscles, we asked what other proteins might work with these molecules. From previous work, *lf* alleles of L-type voltage-gated Ca^{++} channel (L-VGCC)/*egl-19* and ryanodine receptor Ca^{++} channel (RyR)/*unc-68* suppress mutant *unc-103*–induced spontaneous protraction [7,28,29]. Similar to the previously reported interactions between mutant *unc-103* and *egl-19*, *unc-43(sy574)* required wild-type *egl-19* to induce spicule protraction (Table 1). Interestingly, *unc-43(sy574); unc-68(0)* males still spontaneously protract their spicules, suggesting that, in contrast to ERG-like K^+ channel/*unc-103*, CaMKII/*unc-43* is not responding to the influx of calcium via RyR/*unc-68* (Table 1).

L-VGCC/EGL-19 and RyR/UNC-68 have different roles in controlling spicule muscle contraction during male mating. Rhythmic contractions mediated by UNC-68 result in the spicules prodding the hermaphrodite's vulva, whereas tonic contraction mediated by EGL-19 forces the spicules through the vulval slit. The acetylcholine agonist levamisole activates muscle contraction though UNC-68, while EGL-19 is activated by the acetylcholine agonist arecoline (ARE) [8]. Since *unc-43(sy574)* was suppressed by *egl-19(lf)*, but not *unc-68(0)*, the ARE, not the levamisole, stimulatory pathway is perturbed by the *unc-43(sy574)* lesion.

To dissect the CaMKII/*unc-43* pathway, we looked at *unc-43* mutant responses to ARE. Virgin adult males at 1 d old that had not protracted their spicules were placed in various concentrations of ARE and observed for spicule protraction. The concentration at which 90% of wild-type males protract their spicules (EC_{90}) was 579 μM, whereas the EC_{90} of *unc-43(sy574)* males was 268 μM (Figure 2A and 2D). In contrast to wild-type, the dominant gain-of-function allele *unc-43(n498gf)* and the *lf* allele *unc-43(e408)* were greater than ten times more resistant to the drug (Figure 2A and 2D). Thus, the *unc-43(e408)*–encoded kinase displays some gain-of-function properties in the spicule protraction circuit, although in all other behaviors it displays loss-of-function properties. To identify how *unc-43(e408)* might function in the spicule protraction circuit, we sequenced the *unc-43* gene from *e408* animals and found a point mutation that causes a serine to leucine change at amino acid 179 (Figure 1A). This amino

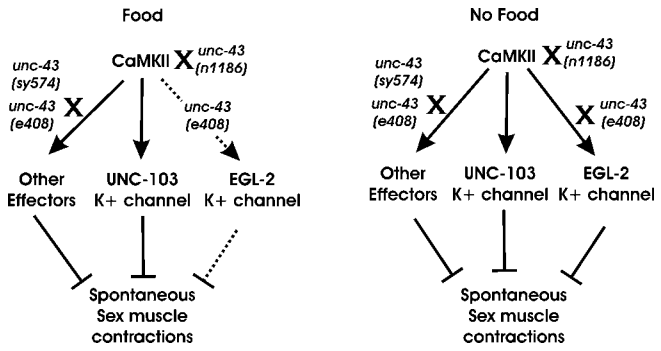

Figure 3. Genetic Interactions between CaMKII, EAG, and ERG-like K^+ Channels in Well-Fed and Food-Deprived Conditions

Arrows represent wild-type CaMKII acts upstream of the K^+ channels and other muscle contraction regulators; bars show that spontaneous muscle seizures are suppressed. An "X" adjacent to an *unc-43* allele name denotes which specific genetic pathway is compromised by the mutant allele. The dashed arrow and bar lines denotes that the interaction between CaMKII and EAG K^+ channels is only seen in the *unc-43(e408)* mutant background.

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acid change is near the substrate recognition site of the catalytic domain, suggesting the *unc-43(e408)* lesion is altering the kinase's interactions with its substrates.

The *n498gf* and *e408* alleles allowed us to determine if CaMKII-induced ARE resistance is mediated by ERG-like K^+ channel/*unc-103*. Previously, *unc-103* was shown to be downstream of *unc-43* in respect to defecation behavior [16]. However, our analyses of *unc-43(sy574)* and *unc-103(0)* suggest that *unc-43* and *unc-103* can act in separate pathways. One explanation for this discrepancy is that CaMKII/UNC-43 can function in multiple pathways to control behaviors, including spicule protraction behaviors (Figure 3). It is likely the *unc-43* alleles used in this report up- or downregulate different facets of spicule protraction regulation. To test if *unc-43* also activates *unc-103* in the spicule circuit, we constructed double mutants of *unc-43(n498gf)* and *unc-43(e408)* with *unc-103(0)* and found they are less resistant to ARE (Figure 2B). Thus, the genetics of the *sy574* allele and the pharmacology of the *n498gf* and *e408* alleles demonstrate that UNC-43 can act concurrently upstream and parallel to UNC-103 (Figure 3). However, the *unc-103(0)*–induced reduction of *unc-43(gf)* and *unc-43(e408)* pharmacology is not complete, indicating there are other factors CaMKII activates to suppress muscle excitability.

To identify other factors that could be activated by CaMKII/*unc-43,* we tested *unc-43's* interaction with the *ether-a-go-go (EAG)* K^+ channel/*egl-2* [30]. We considered EAG K^+ channels because work in *Drosophila* showed that direct phosphorylation by CaMKII upregulates channel activity [15,30,31]. First, we asked if *egl-2* expresses in similar tissues to *unc-43* and *unc-103*. Previous reports showed *egl-2* expression in the sensory neurons and sex muscles of hermaphrodites [30]. We found similar expression in males, including expression in the sex muscles but not neurons in the spicule protraction circuit (Figure 1D and 1E). Next, we asked if *egl-2* acts downstream of *unc-43* by combining *unc-43(lf)* mutations with a gain-of-function *egl-2* allele. *egl-2(n693gf)* was able to reduce the nonsense allele *unc-43(n1186)* and *unc-43(sy574)*–induced spicule protraction, but, interestingly, had no effect on *unc-103(0)*–induced protraction (Table 1). The genetic interaction between *egl-2(n693gf)* and *unc-43(sy574),* but not *unc-103(0),* is consistent with *unc-43(sy574)* disrupting a pathway parallel to UNC-103–mediated regulation (Figure 3).

Since *egl-2(n693gf)* suppressed *unc-43* mutant alleles, we asked if the effects of activated CaMKII/*unc-43* require functional EAG K^+ channel/*egl-2*. We isolated the *rg4* deletion *(0)* allele to address this question and found that *egl-2(0)* animals are superficially wild-type (Table 1). We then combined *egl-2(0)* with *unc-43(n498gf)* and *unc-43(e408)* and found that *egl-2(0)* was able to reduce the ARE sensitivity of both mutations (Figure 2C). Though deletions in *egl-2* and *unc-103* increased drug sensitivity of both *unc-43* mutant backgrounds, neither alone restored it to wild-type levels. We generated triple mutants containing *egl-2(0)* and *unc-103(0)* with *unc-43(gf)* or *unc-43(e408)* to see if removing both K^+ channels increase ARE sensitivity. We found that the EC_{90} of *unc-103(0); unc-43(gf); egl-2(0)* and *unc-103(0); unc-43(e408); egl-2(0)* were 1.2 mM and 483 μM, respectively (Figure 2B–2D). Thus, both ERG-like K^+ channel/*unc-103* and EAG K^+ channel/*egl-2* are required to moderate some of the effects of the activated *unc-43(gf)* allele, and all of the effects of the *unc-43(e408)* allele (Figure 3).

CaMKII and EAG K^+ Channels Are Used during Food Deprivation to Suppress Sex-Muscle Contractions

The pharmacological analyses of genetically activated CaMKII/UNC-43 alleles identified EAG K^+ channel/EGL-2 as a molecule that mediates UNC-43 signaling and acts parallel to ERG-like K^+ channel/UNC-103 (Figure 3). However, the results did not reveal when wild-type sex muscles require regulation by UNC-43 and EGL-2. Indications of when these molecules are used came from our previously reported observations [9].

Under food-deprived conditions, the percentage of ERG-like K^+ channel/*unc-103(0)* males displaying constitutive spicule protraction dropped to 9% from the 33% in food. In contrast, under the same dietary-deprived conditions, the

Table 4. Food Availability Modifies the Expressivity of Abnormal Spicule Protraction

Genotype[a]	Percent Spicule-Protracted Males (*n*)	
	NGM Plates + *E. coli* (OP50)	NGM Plates + No Bacteria
Wild-type	5 (38)	0 (26)
unc-103(0)	36 (44)	9 (23)[b]
unc-43(sy574)	44 (66)	50 (44)
unc-43(n1186)	100 (29)	96 (26)
unc-43(e408)	0 (30)	ND
unc-103(0); unc-43(sy574)	88 (74)	62 (71)[b]
unc-103(0); unc-43(e408)	61 (31)	50 (22)
unc-103(0); egl-2(0)	39 (85)	36 (67)
unc-103(0); egl-2(0) rgEx175 [P_{lev-11}::*egl-2*(+)]	35 (34)	9 (22)[b]
unc-103(0); egl-2(0) rgEx173 [$P_{unc-103E}$::*egl-2*(+)]	39 (28)	4 (23)[b]
unc-103(0); unc-43(e408) rgEx172 [$P_{unc-103E}$::*unc-43*(+)]	38 (45)	13 (40)[b]

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

[b]*p* Value <0.05 compared with NGM plates + *E. coli* of the same genotype; Fischer exact test.

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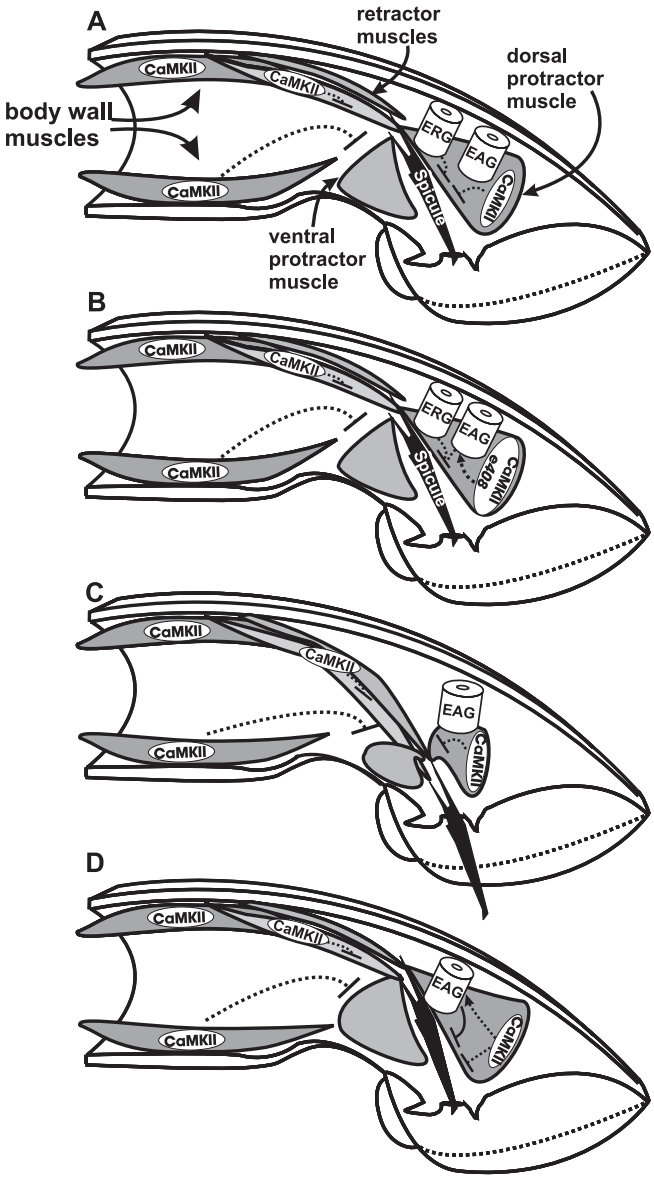

Figure 4. Dietary Deprivation Suppresses Spontaneous Muscle Contractions Using CaMKII and EAG K^+ Channels

Cartoon view of the right half of the male tail. CaMKII is expressed in body-wall muscles, and dorsal and ventral spicule protractor and retractor muscles. EAG and ERG-like K^+ channels are expressed in both dorsal and ventral spicule protractor muscles. CaMKII, EAG, and ERG-like K^+ channels are depicted only in the dorsal protractor muscles for visual clarity.

(A) When males are well-nourished, CaMKII/UNC-43 and ERG-like K^+ channel/UNC-103 act redundantly in body-wall and sex muscles to keep the spicule protractor muscles from spontaneously contracting.

(B) The CaMKII mutant allele *e408* upregulates EAG and ERG-like K^+ channels in the spicule protraction circuit while disrupting the ability of CaMKII to phosphorylate other unknown substrates.

(C) In the absence of functional ERG-like K^+ channel/UNC-103, CaMKII/UNC-43 activity is not sufficient to inhibit spontaneous spicule muscle contraction in 30%–40% of well-nourished males.

(D) When males are food deprived, CaMKII/UNC-43 and EAG K^+ channels compensate for defective ERG-like K^+ channel/UNC-103 and suppress spontaneous muscle contractions.

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percentage of CaMKII/*unc-43(sy574)* and *unc-43(n1186)* males displaying constitutive spicule protraction showed no statistically significant difference from standard food conditions (Table 4). We had also previously demonstrated that perturbing pharyngeal pumping with a missense allele of tropomyosin/*lev-ll(rg1)* can suppress *unc-103(0)*–induced spicule protraction via activity from the pharyngeal neurosecretory motor (NSM) neuron. Consistent with dietary deprivation, the *lev-ll(rg1)* allele also did not reduce the penetrance of the *unc-43(sy574)* phenotype [9]. This suggests CaMKII might be required to mediate the effects of dietary deprivation.

To test if food deprivation requires CaMKII/UNC-43 to suppress ERG-like K^+ channel/*unc-103(0)*–induced spicule protraction, we generated double-mutant combinations between *unc-103(0)* and the *unc-43* alleles *sy574* and *e408*. We tested both alleles because while on food they affected spicule protraction in opposite directions. In food conditions, we found that, like *unc-43(sy574),* the *unc-43(e408)* allele increased the penetrance of *unc-103*–induced protraction. This was not surprising given that the genetic and pharmacology data suggested the *unc-43(e408)*–encoded kinase has reduced functions, but, in the spicule protraction circuit, the mutant kinase upregulates UNC-103 and EAG K^+ channel/EGL-2 activity (Figure 3). Since activated EGL-2 has no effect on the *unc-103(0)* phenotype (Table 1), we hypothesize that the *lf unc-43(e408)* allele acts similar to *unc-43(sy574)* and synthetically interacts with *unc-103(0)* to increase the incidence of constitutive spicule protraction (Figures 3 and 4). In contrast to food conditions, food-depriving *unc-103(0)*; *unc-43(sy574)* males reduced constitutive spicule protraction from 88% to 62% (a percentage similarly displayed by *unc-43(sy574)* single mutants), whereas depriving *unc-103(0)*; *unc-43(e408)* males of food did not change the phenotype. This suggests CaMKII/UNC-43 is required to suppress sex-muscle excitability under food-deprived conditions. In regards to *unc-103(0)*–induced muscle excitability, the *sy574*-encoded kinase can suppress the *unc-103(0)* phenotype. However, under food-deprived conditions, it is unable to suppress its own induced constitutive protraction defect. In contrast, the *e408* mutation can disrupt UNC-43′s ability to transduce food deprivation signals (Figure 3).

To determine if CaMKII/UNC-43 suppresses sex-muscle contraction during food deprivation in ERG-like K^+ channel/*unc-103*–expressing cells, we expressed CaMKII/*unc-43* from the $P_{unc\text{-}103E}$ promoter in the sex muscles and a few head neurons of *unc-103(0); unc-43(e408)* males. We found that rescuing *unc-43* in these cells restored food deprivation–induced suppression of the *unc-103(0)* phenotype (Table 4).

Since our pharmacological and genetic studies suggested that CaMKII/UNC-43 can act through EAG K^+ channel/EGL-2, we asked if these channels are also required for food deprivation–mediated suppression of the ERG-like K^+ channel/*unc-103(0)* phenotype. On food, *unc-103(0); egl-2(0)* males behaved similarly to *unc-103(0)* single mutants (Table 4). However, food deprivation was unable to suppress constitutive spicule protraction in *unc-103(0); egl-2(0)* males. Thus, like UNC-43, EGL-2 is used during food deprivation to reduce sex-muscle excitability (Figure 3). To determine if EGL-2 was functioning in UNC-43– and UNC-103–containing cells, we expressed wild-type *egl-2* cDNA from the *lev-11* and *unc-103E* promoters in *unc-103(0); egl-2(0)* males. We found that

expressing *egl-2* from either promoter restored the ability of food deprivation to suppress *unc-103(0)*–induced spicule protraction. This demonstrates that EAG K^+ channel can function in the same cells as CaMKII to compensate for lack of ERG-like K^+ channel function under dietary deprivation.

Discussion

Intense research in organisms ranging from fungi to vertebrates has uncovered beneficial properties of dietary restriction in delaying aging and increasing cellular stress resistance. However, despite the benefits on physiology, reduction in food accessibility tends to promote food foraging, which if successful, will reduce the effects of dietary restriction. In laboratory vertebrates, multiple studies have shown that reduction in diet can lead to short-term increases in locomotor activity [32–36] and in specialized behaviors such as food hoarding [37–39]. In parallel to vertebrate studies, dietary restriction–induced physiological and behavioral changes have been investigated in the nematode *C. elegans*. The nematode's compact nervous system and musculature provides a simpler and complimentary model to determine how molecules in excitable cells transmit the effects of dietary changes to motor outputs.

When food is reduced or even deprived from *C. elegans,* the nematode displays not only increased longevity and resistance to cellular stresses, but also enhancement in locomotor activity and discrimination between odorants [13,40–43]. These behavioral changes presumably facilitate movement toward a food-related odorant source; but, if that source does not result in food satiation, the animal will move towards an alternative source. In addition to upregulating food-acquiring behaviors, food deprivation also depresses behaviors that are not essential and potentially distracting from feeding. In the absence of food, behaviors such as pharyngeal pumping, defecation, egg-laying, and mate searching are reduced [12,44–46]. When considering the utility of chronic caloric restriction as a mechanism to promote physical wellness in humans, one must consider how brain circuitries normally used in nonfeeding behaviors might be affected. To address this, we used *C. elegans* male mating to identify molecules used to attenuate a behavioral state during dietary deprivation (Figure 4).

Under standard conditions, food-satiated *C. elegans* males display mating behavior upon contact with a hermaphrodite. The male uses sex-specific neurons and muscles located in his tail to recognize a hermaphrodite, scan her body for her vulva, locate and protract his copulatory spicules into the vulva, transfer his genetic material, and withdraw from the hermaphrodite to find additional mates [21]. Deletion of the *unc-103*–encoded ERG-like K^+ channel causes males to display most motor aspects of mating behavior spontaneously; however, since spicule movements provide a facile motor read-out, we have focused on how UNC-103 defects affect the spicule protraction circuit. Prior to mating stimulation, UNC-103 acts in the spicule protractor muscles to keep spontaneous acetylcholine secretion from presynaptic cholinergic neurons (such as the SPC motor neurons) from inducing premature contractions. We found that under well-fed conditions, defective channels cause ~30%–40% of males to protract their spicules constitutively [7]. Under food-deprived conditions, the percentage of males displaying precocious spicule protraction drops to ~9%–11% [9]. This suggests that additional mechanisms act in parallel with UNC-103 to attenuate cell excitability, and in food-limiting conditions, certain aspects of those parallel mechanisms are upregulated.

We isolated the *sy574* allele of CaMKII/*unc-43* gene in the same genetic screen that identified ERG-like K^+ channel/UNC-103 as a regulator of mating behavior [7]. This *unc-43* allele causes ~50% of males to display constitutive spicule protraction; other behaviors in both sexes appear normal. In conjunction with a deletion of *unc-103* [23], 88%–97% of double-mutant males display mating-independent spicule protraction. Similar to the *unc-103* mutant phenotype, defective *unc-43*–induced spontaneous spicule muscle contractions require neurotransmitter secretion from upstream cholinergic neurons such as the SPC neurons (unpublished data). This strongly suggests that CaMKII participates in a regulatory mechanism parallel to ERG-like K^+ channel activity.

CaMKII has a broad expression pattern in neurons and muscles, and has been demonstrated to regulate multiple general behaviors in both sexes of *C. elegans* [16]. Unlike the nonsense allele *unc-43(n1186)* that causes seizures in both sexes and induces 100% spicule protraction in males, the *sy574* mutant kinase must be grossly functional in many behavioral circuits, but has reduced activity for regulating sex-muscle contraction. The *sy574* lesion induces two amino acid changes that map to the kinase domain and the self-association domain of UNC-43. However, the change in the self-association domain might not be relevant; a truncated *unc-43* transgene lacking the self-association domain is sufficient to rescue the *sy574* spicule defect. The G170E change in the kinase domain of the *sy574* allele is located in an amino acid residue that is conserved in CaMKII of diverse species. The structure of the UNC-43 kinase domain has been solved [47], and the G170E change lies in a structural region of the kinase domain that has not been intensely dissected and characterized, but could be involved in substrate recognition. The differences in phenotypic severity between the nonsense *unc-43(n1186)* and missense *unc-43(sy574)* alleles suggest that the nonpolar-to-acidic amino acid substitution at position 170 might disrupt the efficiency of how the UNC-43(*sy574)* kinase phosphorylates certain effectors in the spicule protractor muscles.

CaMKII/UNC-43 has been previously shown to upregulate the activity of ERG-like K^+ channel/UNC-103 in the defecation circuit [16,23]. We show through pharmacology and genetics that this type of regulation also occurs in the spicule protraction circuit, but we do not believe this regulation is abrogated by the *sy574* allele. Although *unc-43(sy574)* males superficially display the same behavioral defects as *unc-103(0)* males, *unc-43(sy574)*–induced spicule protraction can not be suppressed by food deprivation. A circuit that acts in suppressing *unc-103(0)*–induced sex-muscle seizures incorporates the activity of the pharyngeal muscles and the pharyngeal NSM neurons. In standard food conditions, a missense mutation in tropomyosin/*lev-11* can phenocopy the effects of food deprivation, and partially suppress the *unc-103(0)* phenotype. The mutant tropomyosin reduces pharyngeal pumping, but the feeding defect is masked by activity from the pharyngeal NSM neurosecretory neurons. When the NSM neurons in *lev-11* mutants are laser ablated, pharyngeal

pumping rate decreases, and concurrently, *unc-103(0)*–induced sex-muscle seizures are no longer suppressed. The NSM neurons are hypothesized to sense changes in pharyngeal pumping rate and secrete a factor(s) that attenuates muscle excitability, thus suppressing the effects of defective UNC-103 function. Interestingly, similar to depriving males of food, the *lev-11* mutation, which can suppress *unc-103(0),* has no effect on *unc-43(sy574)*–induced spicule protraction [9].

Since the CaMKII/*unc-43(sy574)* phenotype is not suppressed by food deprivation or pharyngeal muscle–NSM neuron interaction, we suspected activated wild-type UNC-43 might be required to attenuate cell excitability during nonoptimal food conditions. The strong *lf unc-43(e408)* allele was used to test if CaMKII is required during food deprivation. The *e408* mutation encodes a serine to leucine change at amino acid 179 nine residues from the glycine that is changed in the *unc-43(sy574)* allele. The *unc-43(e408)* allele induces pleiotropic behavioral abnormalities such as seizures and inhibited egg-laying, suggesting that UNC-43*(e408)* kinase activity is severely reduced. In contrast to the nonsense and *sy574* mutations, under standard food conditions, the S179L change causes the UNC-43*(e408)* kinase to decrease the excitability of male sex muscles via EAG family K^+ channels (Figure 4B). The S179L change might reduce effector interactions so that the kinase cannot phosphorylate many of it substrates, except for those that directly or indirectly regulate K^+ channels in the sex muscles. Thus, the *unc-43(e408)* allele disrupts kinase function in a manner different from the *unc-43(n1186)* nonsense allele, which has been shown to severely reduce the amount of CaMKII present in hermaphrodites [16]. Under food deprivation conditions, males containing the UNC-43*(e408)* kinase are not able to significantly suppress *unc-103(0)*–induced muscle seizures. The inability of *unc-43(e408)* to reduce *unc-103(0)*–induced muscle seizures suggests that the S179L substitution, which reduces CaMKII function in attenuating excitability of many behaviors, also disrupts how food deprivation attenuates the excitability of male muscles. Due to the broad effects of food deprivation, we hypothesize that multiple pathways are used to suppress sex-muscle excitability in response to low food conditions. Since neither *unc-103(0); unc-43(e408)* double-mutant or *unc-43(n1186)* single-mutant males are significantly suppressed by food deprivation, it is possible that the majority of these pathways require CaMKII. However, since there is a slight reduction in each case, as well as a significant reduction in *unc-103(0); unc-43(sy574)* males, there might be other pathways working in parallel that are CaMKII independent. Taken together, these data suggests that wild-type CaMKII is required to decrease cell excitability during dietary deprivation conditions, and the mechanism of regulation differs from conditions where food is available.

We propose that calcium influx activates CaMKII and EAG K^+ channels to depress cell excitability, which can compensate for ERG-like K^+ channel dysfunction. In *C. elegans,* the ortholog of EAG is encoded by the gene *egl-2* [30]. Synaptic plasticity studies in *Drosophila* have shown that neuronal CaMKII directly phosphorylates EAG K^+ channels. This phosphorylation is presumed to maintain or enhance EAG K^+ channel activity during calcium influx and after intracellular calcium has dropped to basal levels [14,15,31]. In *C. elegans,* EGL-2 is coexpressed with UNC-103 and CaMKII/UNC-43 in the spicule muscles. Under standard food conditions, the *egl-2(0)* mutation induces no obvious abnormal defect either by itself or in conjunction with *unc-103(0)* in regulating spicule muscle contraction. Similarly, the gain-of-function *egl-2(n693)* mutation, which hyperpolarizes sensory neurons, egg-laying, and defecation muscles, also does not enhance or suppress *unc-103(0)*–induced seizures. Taken together, the genetic data suggest that under standard food conditions, EGL-2 K^+ channels, although present in the spicule muscles, are not active. However, similar to males containing the severe *lf unc-43(e408)* mutation, deletion of *egl-2* reduces the ability of food deprivation to suppress *unc-103(0)*–induced muscle seizures.

Our data suggest that under food deprivation conditions, calcium influx activates CaMKII and EAG K^+ channels to depress cell excitability, which can compensate for ERG-like K^+ channel dysfunction. We speculate that in well-fed wild-type males, ERG-like K^+ channels act in parallel with a CaMKII-regulated pathway(s) to attenuate mating behavior until specific cues are presented (Figure 4A). If one of these mechanisms is disrupted, the other can compensate in a percentage of animals (Figure 4C). In the absence of food, CaMKII and EAG K^+ channels additionally act to hyperpolarize the sex muscles, thus suppressing mating behavior so that the male will forage for food, rather than mate (Figure 4D). We have demonstrated that one can take advantage of this dietary deprivation pathway to suppress spontaneous muscle seizures caused by defective ERG-like K^+ channels. We suspect that similar mechanisms that occur during food deprivation in *C. elegans* males also function in neurons and muscles in higher animals. This is consistent with many studies that have shown that caloric restriction can reduce seizure susceptibility in epileptic mouse models [48–50]. Although the exact molecules will differ between neuronal types in various excitable cell circuitries, co-opting endogenous parallel regulatory pathways to compensate for ion channel dysfunction should be a feasible general strategy.

Materials and Methods

Strains. All strains contain *him-5(e1490)* (LGV) [51] and were maintained as described in [52]. The following strains were used. LGI: *lev-11(rg1)* [9]; LGIII: *unc-103(n1213)* [53] and *pha-1(e2123)* [54]; LGIV: *unc-43(e408)* and *unc-43(e266)* [52], *unc-43(n1186), unc-43(n1179), unc-43(n498)* [53], *unc-43(sa200)* [45], and *egl-19(n582)* [55]; and LGV: *unc-68(r1158)* [28]; and *egl-2(n693)* [56]. The strain CB4856 was used for single nucleotide polymorphism mapping [17].

Identification of *sy574*. The isolation of *sy574* has been previously described [7]. *sy574* animals were out-crossed five times. *sy574* contains two missense changes: *sy574*A changes the sequence CACGGATTT to CACGAATTT, and *sy574*B changes GCCGCGTGT to GCCGTGTGT. *unc-43* was also sequenced in the strain used for mutagenesis, PS1385, and no mutations were found. In *unc-43(e408),* the *e408* allele changes the sequence TTGTCGCCA to TTGTTGCCA.

Generation of the *egl-2* deletion allele *rg4*. *egl-2(rg4)* was generated by trimethylpsoralen mutagenesis of *egl-2(n693gf) him-5* [57]. After mutagenesis, we selected worms that displayed normal egg-laying behavior. PCR analysis using primers that annealed to internal exons of *egl-2* were used to screen lines for deletions in the *egl-2* locus. *egl-2(rg4)* includes exons 1–7, but does not include the pore region or the *egl-2(693gf)* mutation [30]. The *egl-2(rg4)* deletion ends before the start of the next gene *pme-5*. *egl-2(rg4)* animals were out-crossed four times.

Assaying spontaneous spicule protraction phenotype. L4 males were separated and allowed to develop into adults overnight on NGM plates seeded with OP50. Generally, 20–30 worms were analyzed per plate. The adult males were scored as positive for displaying spontaneous spicule protraction if at least one spicule protruded from the cloaca.

Pharmacology. L4 males were isolated and allowed to mature overnight on NGM plates. The next day, males that did not display constitutive spicule protraction were placed, five at a time, in solutions of ARE (Indofine Chemical Company, http://www.indofinechemical.com) in Pyrex, round-bottom, three-well titer plates. The males were observed for 5 min and scored if their spicules remained protracted for at least 10 s. Curve fits and EC_{90} values were determined using GraphPad Prism (version 4.03; http://www.graphpad.com).

Assay for food deprivation on the protraction constitutive phenotype. We used 10-mm NGM plates with an 8-M glycerol ring surrounding the outer edge of the plate. The 8-M glycerol ring served as a repellent during the assay to keep males from crawling up the inside edge of the plate; the 8-M glycerol ring induced no other significant changes in male behavior (unpublished data). To measure the effects of food deprivation, we separated late L4–stage males to a clean NGM plate with no *Escherischia coli* OP50 and allowed them to crawl away from any OP50 transferred. Males were then picked up by mouth pipette, washed with M9 buffer, and transferred to a clean NGM plate with the glycerol ring described above. As a control, sibling males of the same stage were placed on 10-mm NGM plates seeded with OP50. Males from both the control and experimental plates were then assayed 15–20 h later for the constitutive protraction phenotype.

Plasmids used in this study. The details of the construction of the plasmids used in this study are listed in Text S1 and Table S1. Plasmids containing *unc-103* genomic DNA were created as previously described [23]. The plasmids pBL58, pBL70, pBL69, pBL71, pBL72, and pBL80 contain the *unc-43*g cDNA expressed from the *hsp-16*, *aex-3, lev-11, unc-103*E, *tnt-4,* and *acr-8* promoters, respectively. The plasmids pTG44 and pTG46 contain the *egl-2* cDNA expressed from the *unc-103*E and *lev-11* promoters, respectively. pBL68 contains *unc-43* cDNA lacking the self-association domain expressed from the *lev-11* promoter. pBL66 contains CFP expressed from the *gtl-1* promoter. pLR16 contains yellow fluorescent protein (YFP) expressed from the *egl-2* promoter.

Transgenics. Worms expressing *unc-103* transgenic lines were previously described [23]. L4 males were isolated and scored the next day to determine the number of males that displayed protruding spicules. To obtain *unc-43* transgenic lines, DNA was injected into *unc-43(sy574); him-5(e1490)* hermaphrodites out-crossed three times using standard protocols [58]. The injection mixtures were created as follows: the injected concentrations of the *unc-43* plasmids, pBL69 (50 ng/μl), pBL70 (50 ng/μl), pBL72 (50 ng/μl), pBL58 (26 ng/μl), and pBL71 (10 ng/μl) were combined with the appropriate amount of pUC18 to bring the total concentration of DNA to 180 ng/μl. In addition, each mixture contained 20 ng/μl of pBL66 that was used as a marker to identify transgenic lines. pBL68 was injected along with pUC18 (92 ng/μl) and the *pha-1*–rescuing plasmid pBX1 (100 ng/μl) [59] into *pha-1(e2131); unc-43(sy574); him-5(e1490)* at a concentration of 23.6 ng/μl. F1 hermaphrodite progeny with CFP expression in their intestines were selected. For each injection, three to five lines were analyzed; one representative line is shown in the tables.

Males from the transgenic lines were scored for spontaneous spicule protraction in the following manner: 6 L4 hermaphrodites for each line were placed on individual NGM + OP50 plates. Only first-generation L4 males of the isolated hermaphrodites were picked, allowed to mature to adults overnight at 20 °C, and then scored for constitutive spicule protraction. For males containing UNC-43 cDNA expressed from the heat-shock promoter, late-stage L4 males were heat-shocked at 33 °C for .5 h and then scored for the instance of spicule protraction the next day.

Supporting Information

Table S1. Primers

Found at doi:10.1371/journal.pgen.0030156.st001 (23 KB DOC).

Text S1. Supplemental Experimental Procedures

Found at doi:10.1371/journal.pgen.0030156.sd001 (45 KB DOC).

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