Cell Excitability Necessary for Male Mating Behavior in *Caenorhabditis elegans* Is Coordinated by Interactions Between Big Current and Ether-A-Go-Go Family K⁺ Channels

Brigitte LeBoeuf and L. Rene Garcia¹

Howard Hughes Medical Institute, Department of Biology, Texas A&M University, College Station, Texas 77843

ABSTRACT Variations in K⁺ channel composition allow for differences in cell excitability and, at an organismal level, provide flexibility to behavioral regulation. When the function of a K⁺ channel is disrupted, the remaining K⁺ channels might incompletely compensate, manifesting as abnormal organismal behavior. In this study, we explored how different K⁺ channels interact to regulate the neuromuscular circuitry used by *Caenorhabditis elegans* males to protract their copulatory spicules from their tail and insert them into the hermaphrodite's vulva during mating. We determined that the big current K⁺ channel (BK)/SLO-1 genetically interacts with *ether-a-go-go* (EAG)/EGL-2 and EAG-related gene/UNC-103 K⁺ channels to control spicule protraction. Through rescue experiments, we show that specific *slo-1* isoforms affect spicule protraction. Gene expression studies show that *slo-1* and *egl-2* expression can be upregulated in a calcium/calmodulin-dependent protein kinase II-dependent manner to compensate for the loss of *unc-103* and conversely, *unc-103* can partially compensate for the loss of SLO-1 function. In conclusion, an interaction between BK and EAG family K⁺ channels produces the muscle excitability levels that regulate the timing of spicule protraction and the success of male mating behavior.

THE neuronal and muscular networks that execute behavioral responses to stimuli are ultimately maintained through cross talk by the various molecular signaling components. This signaling is essential for fine-tuning cell excitability levels to generate appropriate responses and is partially accomplished by a variety of K⁺ channels. Organisms have a large number of K⁺ channels that have multiple isoforms and ancillary subunits (Salkoff *et al.* 2006; Torres *et al.* 2007; Fodor and Aldrich 2009). In excitable cells, a few K⁺ channel types are responsible for the maintenance of depolarization; the rest are proposed to be modifiers, fine-tuning the particular cell or cell type for its specific function (Bargmann 1998; Santi *et al.* 2003).

The importance of these modifiers is highlighted by the multitude of human disorders that exist as a result of defective K⁺ channels. These disorders include epilepsy, which can be caused by dysfunction of a variety of channels

(Schmitt *et al.* 2000; Du *et al.* 2005), long Q-T syndrome, one form of which can be caused by mutations in the HERG gene and leads to cardiac arrhythmias and sudden death (Sanguinetti *et al.* 1995), hypertension caused by kidney malfunction (Grimm and Sansom 2010), and erectile dysfunction (Werner *et al.* 2005).

The K⁺ channels, which fine-tune cell excitability, need to work together. When there is a defective or absent channel, the remaining K⁺ channels might compensate in an incomplete manner. However, the structure and function of the compensating channels likely differ from the conventional one, resulting in imperfect regulation of cell excitability. Determining how defective K⁺ channels change the dynamics of the remaining K⁺ channels will provide insights into how the integration of their functions maintains appropriate excitability levels.

To address how the regulation of cell excitability and behavior is coordinated by fine-tuning K⁺ channels, we used *Caenorhabditis elegans* male mating as a model system. The stereotyped steps of this behavior include contact response, backward locomotion and vulval scanning, turning, vulva sensing, repetitive spicule insertion attempts, complete spicule

penetration, and sperm transfer. Males utilize a pair of copulatory spicules to breach the hermaphrodite vulva. The spicules must be maintained inside the male tail prior to mating and during mating until vulva penetration (Liu and Sternberg 1995). We have previously identified two K⁺ channels, *ether-a-go-go* (EAG) and EAG-related gene (ERG), that inhibit protraction until the vulva has been breached (Garcia and Sternberg 2003; LeBoeuf *et al.* 2007). However, these two K⁺ channels do not account for all the regulation necessary to coordinate spicule protraction. In this article we identify the big current (BK) SLO1 K⁺ channel as an additional regulator of male sex muscle excitability. Since these three K⁺ channels function in the mating circuit to regulate behavior, we explored their effects on each other and elucidated how their interplay maintains the cell excitability that allows a male to successfully sire progeny.

Materials and Methods

Strains and culture methods

The following strains were used in this study: *unc-103(n1213)* (Park and Horvitz 1986), *unc-103(sy557)* (Garcia and Sternberg 2003), and *pha-1(e2123)* (Schnabel and Schnabel 1990) on LGIII; *unc-43(sy574)* (LeBoeuf *et al.* 2007) on LGIV; *him-5(e1490)* (Hodgkin *et al.* 1979), *slo-1(js379)* (Wang *et al.* 2001), *slo-1(rg432)* (this work), *egl-2(rg4)* (LeBoeuf *et al.* 2007), and *egl-2(n693)* (Reiner *et al.* 1995) on LGV. *C. elegans* is a naturally hermaphroditic species with a low incidence of males; to generate a larger percentage of males, strains in this study contained the *him-5(e1490)* allele. Animals were maintained on NGM plates seeded with *Escherichia coli* strain OP50 at 20° (Brenner 1974).

Protraction constitutive assay

A total of 20–30 virgin L4 males were isolated on NGM plates with OP50. The males were allowed to develop into adults overnight and scored as positive for spicule protraction if at least one spicule partially extended from the cloaca. *P* values were determined using GraphPad Prism (version 4.03).

Identification of the *slo-1(rg432)* mutation

We sequenced all of *slo-1*, including the promoter region, from the genomic DNA of an *unc-103(n1213)*; *egl-2(rg4)* strain with a lower-than-normal instance of the protraction constitutive (Prc) phenotype. We discovered that *rg432* changes the sequence TGACATTTATATTATCATTTT to TGACATTTATTTATCATTTT in the intronic region between exons 11 and 12 (exons numbered according to Wang *et al.* 2001).

Plasmid constructs

pBK1 contains the *slo-1* promoter and isoform *slo-1(A2;B0;C1;D0)* tagged with GFP and was provided by Michael Nonet (Washington University, St. Louis). To express *slo-1(A2;B0;C1;D0):GFP* in specific tissues, we removed the *slo-1* pro-

motor from pBK1 and added the *XbaI* restriction site in front of the cDNA via single-site mutagenesis with primers FpBK1XbaI and pBK1r to create plasmid pBL161. pBL161 was then cut with *XbaI*, blunt-ended, and Gateway vector conversion reading frame cassette (RfC) A (Invitrogen, Carlsbad, CA) was ligated into the site to create plasmid pBL176. The tissue-specific constructs were created using LR clonase (Invitrogen) to recombine the pBL176 destination vector with the vectors containing the tissue-specific promoters. pBL176 was recombined with pLR35 (Paex-3, pan-neuronal) (LeBoeuf *et al.* 2007), pLR21 (Punc-103E, sex muscles) (Reiner *et al.* 2006), and pLR92 (Pacr-8, body-wall muscles) (LeBoeuf *et al.* 2007) to create plasmids pBL179, pBL180, and pBL181, respectively.

We cloned *slo-1(A2;B0;C0;D0)* into plasmid pSX322YFP to generate pBL185cc. *slo-1(A2;B0;C0;D0)* was cloned from PCR-amplified cDNA using primers FslolcDNASphI and RslolcDNAXbaI. The cDNA was generated from poly(A) RNA using Qiagen's (Valencia, CA) Oligotex mRNA Mini kit and Invitrogen's SuperScript First-Strand Synthesis system for RT-PCR. Both the plasmid and PCR product were cut with *SphI* and *XbaI* and ligated together to generate pBL185cc. In this manner, we cloned nine different *slo-1* full-length cDNA isoforms, and we discovered a new exon located between exons 2 and 3. We cut pBL185cc with *NheI* and ligated Gateway RfC A (Invitrogen) in front of *slo-1(A2;B0;C0;D0)* to create plasmid pBL192. pBL192 contained point mutations in the *slo-1* cDNA that changed amino acids. To correct the *slo-1* sequence, we cut pBL192 and a plasmid containing *slo-1(A2;B0;C0;D0)* provided by Brandon Johnson and Miriam Goodman (Stanford University, Palo Alto, CA) with *BseRI*. The short fragment from digested *slo-1(A2;B0;C0;D0)* was ligated to the long fragment from digested pBL192 to create pBL224. We recombined a PCR fragment of the *slo-1* promoter generated with primers Fslolp and Slo1pr from N2 genomic DNA with pDG15 using BP clonase (Invitrogen) to generate plasmid pBL153 (the same promoter region reported in Wang *et al.* 2001). We performed an LR reaction between pBL153 and pBL224 to generate plasmid pBL226.

A plasmid containing a Gateway RfC, *slo-1(A1;B0;C0;D0)*, and GFP was created by cutting pBL176 and a plasmid containing *slo-1(A1;B0;C0;D0)* provided by Miriam Goodman with *StuI* and *HpaI*. The long restriction enzyme product from cutting pBL176 was ligated to the short restriction enzyme product from *slo-1(A1;B0;C0;D0)* to generate pBL198. pBL198 was recombined with pLR21 and pBL153 via LR clonase to create plasmids pBL204 and pBL198, respectively.

Plasmids containing mutated *unc-103* genomic DNA were generated using single-site mutagenesis on plasmid pLR67, which contains *unc-103* genomic DNA plus Gateway RfC C.1 (Invitrogen) (Reiner *et al.* 2006). The primers psy557A and phosrevsy557A were used to create pLR74 containing the sy557A mutation, and the primers psy557B and phosrevsy557B were used to create pLR75 containing the sy557B

mutation. pLR74 was recombined with pLR21 and pLR28 (Punc-103F, sex neurons) (Reiner *et al.* 2006) using LR clonase to create plasmids pBL35 and pBL36, respectively. pLR75 was recombined with pLR21 and pLR28 using LR clonase to create plasmids pBL34 and pBL37, respectively.

pTG44 containing the *unc-103E* promoter driving *egl-2* cDNA was constructed as previously described (LeBoeuf *et al.* 2007). We added the *n693gf* mutation by performing single-site mutagenesis on pTG44 with primers *fegl2n698gf* and *regl2698gf* to create plasmid pBL111 (LeBoeuf *et al.* 2011).

pBL160 was created via an LR reaction between pBL153 (Pslo-1) and pLR186 (Invitrogen Gateway Rfc C.1:DsRed1-E5) (LeBoeuf *et al.* 2011).

Transgenics

Plasmids containing the construct of interest were injected into 1-day-old adult hermaphrodites of the appropriate strain following standard procedures (Mello *et al.* 1991). Either 50 ng/μl pBL66 or 100 ng/μl pBX1 were used as a transgenic marker (Granato *et al.* 1994; LeBoeuf *et al.* 2007). pUC18 was used as carrier DNA to complete each injection mixture to a final concentration of 200 ng/μl. For each plasmid injected, concentrations were as follows: 20 ng/μl was used for pBL34, pBL35, pBL36, pBL37, pBK1, pBL180, pBL179, pBL181, and pBL199 and 50 ng/μl was used for pBL111, pBL160, pBL226, and pBL204. Once stable transgenic lines were obtained, males from two or more independent transgenic lines were scored for the Prc phenotype following the procedure previously described.

Integration

To obtain a stable expression line to measure fluorescent levels in males carrying Pslo-1:DsRed1-E5, the transgene was integrated using trimethylpsoralen following standard procedures (Anderson 1995) to create transgenic line *rgIs2*.

Image acquisition and quantification

Males carrying the *rgIs2* transgene were isolated at mid-L4. Images were either taken immediately, or the males were allowed to develop into adults on NGM plates containing *E. coli* OP50 for 1 or 2 days. Males were immobilized on 10% Noble agar (dissolved in water) pads with Polybead polystyrene 0.1-μm microspheres (Polysciences, Warrington, PA) and covered with a cover slip. Images were taken using an Olympus BX51 microscope with a ×40 objective. The image signal was split into two channels using the Dual View Simultaneous Imaging system by Photometrics (Tucson, AR). Pictures were taken by the Hamamatsu EM-CCD digital camera ImagEM using HC Image (version 2.1.1.0) (Bridgewater, NJ). All images were taken with the gain, sensitivity, and exposure time held constant. The one exception is at the 2-day time point, when the red channel was overexposed at the standard settings. One image was taken at the standard settings and a second image was taken when the gain was lowered so the red channel was no longer overexposed. Data

from both images were used to obtain the mean gray level for the red channel (see below).

Images were analyzed using Hamamatsu Simple PCI (version 6.6.0.0). A region of interest (ROI) was placed over the male tail in both the red and green channels and in the background of both the red and green channels to get the background fluorescent level. The ROI was held to a constant size using the “ROI clone” tool. The mean gray level was recorded for each ROI, and the background fluorescence was subtracted. For 2 day-old adult males, the mean gray level was determined for the green channel under standard settings as well as both the green and red channel with the gain lowered. Since the green channel was not overexposed in either image, a ratio between the high gain and low gain image was obtained. This ratio was then combined with the low gain red mean gray level to approximate the standard gain red mean gray level. The analyzed images were not modified in any way. Statistical analysis was performed using GraphPad Prism (version 4.03).

Quantitative real-time PCR

Each strain of worms was cultivated on six 100-mm NGM plates containing *E. coli* OP50. Worms were harvested before they starved by washing the plates with M9 buffer. Worms were collected at 1000 rpm and the M9 and *E. coli* were removed. The worms were washed with an additional 1 ml of M9 to remove additional *E. coli*. For experiments where hermaphrodites were segregated from males, worms were first placed on a 20-μM Nitex nylon filter to remove larva, and then on a 35-μM Nitex nylon filter to separate males and hermaphrodites (Sefar Filtration, Depew, NY). Worms were divided into four 1.5-ml microcentrifuge tubes and 250 μl Tri Reagent (Sigma-Aldrich, St. Louis) and 0.5 mm zirconium oxide beads (Next Advance, Cambridge, MA) were added. The Bullet Blender (Next Advance) set for 3 min at speed 8 was used to break open the worms. The debris, Tri Reagent, and beads were spun down for 1 min at top speed in a microcentrifuge. The Tri Reagent and RNA were moved to a fresh RNase-free 0.5-microcentrifuge tube. Ten microliters of glycogen and 50 μl of chloroform were added, and the mixture was shaken by hand and centrifuged for 10 min at 12,000 rpm to separate the layers. The aqueous layer was transferred to a fresh 0.5-RNase-free microcentrifuge tube. Two ethanol precipitations were performed to remove any salts that might interfere with the ability to separate poly(A) RNA from total RNA. The quality of the total RNA was determined using a 2% agarose gel and the quantity was measured using a spectrophotometer. Poly(A) RNA was obtained using Ambion's Poly(A) Purist kit (Applied Biosystems, Austin, TX). cDNA was made using the SuperScript First-Strand Synthesis system for RT-PCR (Invitrogen) with 200 ng of starting poly(A) RNA from each sample and replicated at least one time. Reference genes were determined as done in Vandesompele *et al.* (2002) and Hoogewijs *et al.* (2008). Thirteen reference genes were considered: *act-1*, *gpd-3*, F23B2.13, *rrn-1.1*, *cdc-42*,

Y45F10D4F.3, *ama-1*, *csq-1*, *elf-3.C*, *mdh-1*, *gpd-2*, *pmp-3*, and *tba-1*. geNorm was used to identify the most stable reference genes (<http://medgen.ugent.be/~jvdesomp/genorm/>). The two most stable reference genes for comparison across K⁺ channel mutants were *act-1* and *mdh-1*. Amplification was performed using Bio-Rad's ssoFast EvaGreen on a Bio-Rad CFX96 Real-Time Detection system (Hercules, CA). *act-1* and *egl-2* were amplified using the primers reported in LeBoeuf *et al.* (2011). *mdh-1*, *cdc-42*, and *pmp-3* were amplified using the primers reported in Hoogewijs *et al.* (2008). *unc-103* was amplified using primers 2qPCRunc-103F and 2qPCRunc-103R. *slo-1* was amplified using primers F1-qPCRslol1ex11 and qPCRslol1ex12-1R. The primers for *slo-1* A1 were Fslol1alex9 and Slo1alex9r. The primers for *slo-1* A2 were Fslol1a2ex10 and Slo1a2ex10r. The primers for *slo-1* B0 were Fslol1b0 and Slo1b0ex14r. The primers for *slo-1* B1 were Fslol1b1 and Slo1b1r. The primers for *slo-1* C0 were Fslol1c0 and Slo1c0r. The primers for *slo-1* C1 were Fslol1c1 and Slo1c1r. The primers for *slo-1* D0 were Fslol1d0 and Slo1d0r. The primers for *slo-1* D1 were Fslol1d1 and Slo1d1r2. The primers for *acr-18* were qPCRacr-18F and qPCRacr-18R. The primers for *gar-3* were 2qPCRgar-3F and 2qPCRgar-3R. The primers for *unc-29* were qPCRunc-29F and qPCRunc-29R. The primers for *unc-43* were qPCRunc-43F and qPCRunc-43R. The primers for *cat-2* were qPCRcat-2F and qPCRcat-2R. The primers for *let-363* were qPCRlet-363F and qPCRlet-363R. The primers for *sir-2.1* were 2qPCRsir-2.1F and 2qPCRsir-2.1R. Samples were diluted 1:10 for all K⁺ channel sequences, 1:1000 for *mdh-1*, and 1:10,000 for *act-1*. All C(t) values were between 20 and 30. Each sample was done in triplicate, and each experiment was repeated at least once from newly made cDNA. Expression levels of each gene of interest were normalized to *act-1* and *mdh-1* and then reported as relative gene expression levels using CFX Manager Software Data Analysis (Bio-Rad version 1.6) according to the C_T method (Livak and Schmittgen 2001). Statistical analysis was performed using GraphPad InStat (version 3.06). The results reported in Figure 3 are from one representative experiment for each gene tested.

Primer designations

The primer designations are as follows:

FpBK1XbaI: 5'-TCTAGATGGGCGAGATTTACTCGCCTTCGCA
pBK1r: 5'-GCATGCAAGCTTATTTCAITTTCCAAGTTGTTAGC
GTATC
Fslol1p: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCAG
GAAGATTTATAGAGTCTTCTGATGAATT
Slo1pr: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCGT
GTCGACGAAGGGTCCAC
Fslol1cDNASphI: 5'- CGCGGCATGCGCTAGCATGGGCGAGA
TTTACTCGCCTTCGAGTCCG
Rslo1cDNAXbaI: 5'- CGCGTCTAGAAAAGTGTCGTTTGCCC
GGCTCGTACTCCAGTC
psy557A: 5'-GGATCCAGGAAAAATTGCCACGAATTACTTC

phosrevsy557A: 5'-GACACCACCTGACACGCCTATTATTGTGG
psy557B: 5'-CATTTGGTTGGCATGTATACGGTGAGATTTTAAG
phosrevsy557B: 5'-TGCTATAAGTGCAAATGTAGCCATAAGAAG
2qPCRunc-103F: 5'-TTCAGTGTCTCCAACAACGG
2qPCRunc-103R: 5'-TGTTCTGCATTCGTTCCATTG
F1-qPCRslol1ex11: 5'-CTGGTGTAAGCAGTGTTCATGAC
qPCRslol1ex12-1R: 5'-GGCCGCTGTCGAGTGTTCG
Fslol1alex9: 5'-TCTCCTCACACCCCTCTCTG
Slo1alex9r: 5'-GCAGCTTCTGGAAATGACATATTC
Fslol1a2ex10: 5'-TCACAAACCACACCGGACTGG
Slo1a2ex10r: 5'-TCGACAGCTTCAGGAAAAGTCATTC
Fslol1b0: 5'-GGCCCGTGCAAGAGCCA
Slo1b0ex14r: 5'-CTCTGTTGATTAATCATCCTTAGCTG
Fslol1b1: 5'-GGCCCGTGATTAATTCGGACTTTG
Slo1b1r: 5'-CTCTGTTGATTAATCATCCTTAGCTG
Fslol1c0: 5'-ATCAACAGAGGCCATCTTCCG
Slo1c0r: 5'-GTACTGTCATATTTTCATGCTTTGG
Fslol1c1: 5'-GAAATCCCTTCGATTTGCCTATG
Fslol1d0: 5'-AAGCCGTCCAAATAAATATGAACGGG
Slo1d0r: 5'-CCCAGTCCTTGGCTTCTGTG
Slo1d1r2: 5'-GTCCTTGGCCCTGCGGTCC
qPCRacr-18F: 5'-GAGATCGGATCACCGGAAAC
qPCRacr-18R: 5'-CACTTCCACCACCGGTTATC
2qPCRgar-3F: 5'-CAATTCGGACACCTATACAGTTTTG
2qPCRgar-3R: 5'-GCTCGTCAATCTCACTATTACAATC
qPCRunc-29F: 5'-AGTACTGATGCTCTACGAGC
qPCRunc-29R: 5'-ATACGTGAGGCGCGGAAAAAC
qPCRunc-43F: 5'-CCAAGTGACACAACAATGCTC
qPCRunc-43R: 5'-ATGAACACACACCCAACGGC
qPCRcat-2F: 5'-ACTACACGGAGGAAGAGCAC
qPCRcat-2R: 5'-AAGTGAGGCCAGGAAGTCG
qPCRlet-363F: 5'-ACGTCAGAATATGGCTGGTG
qPCRlet-363R: 5'-AAGTGGTTTCAGGTGGAGTTG
2qPCRsir-2.1F: 5'-CGAGACACATTGCGAAAGTTC
2qPCRsir-2.1R: 5'-TGCAAGTCCAGATGTATCGTG.

Results

A BK channel/*slo-1* mutation results in increased male mating circuit excitability

C. elegans males possess copulatory spicules that must be held inside their tail until the hermaphrodite vulva is breached. Protractor and retractor muscles are attached to the base of the spicules and control their proper position. Once the appropriate mating cues have been received and integrated by both neurons and muscles, the protractors contract, forcing the spicules out of the tail and into the vulva (Sulston *et al.* 1980; Liu and Sternberg 1995; Liu *et al.* 2011). Permanent spicule protraction in the absence of mating cues interferes with successful sperm transfer and thus reproduction. This abnormal spicule phenotype is referred to as **Protraction constitutive (Prc)** and arises when mechanisms that tightly regulate the spicule protraction circuit begin to fail. The percentage of Prc males in a population

Table 1 Spicule protraction induced by mutations in K⁺ channels

Genotype	% spicule protracted (n)	P value, Fisher's exact test
Wild type	12 (42)	
<i>egl-2(0)</i>	0 (37)	
<i>unc-103(0)</i>	41 (80)	0.0005 to <i>unc-103(sy557)</i>
<i>unc-103(0); egl-2(0)</i>	83 (46)	<0.0001 to <i>unc-103(0)</i>
<i>slo-1(lf)</i>	70 (201)	
<i>unc-43(sy574)</i>	38 (65)	
<i>unc-43(sy574); slo-1(lf)</i>	96 (56)	<0.0001 to <i>slo-1(lf)</i>
<i>unc-103(0); slo-1(lf)</i>	51 (64)	<0.05 to <i>slo-1(lf)</i>
<i>egl-2(0) slo-1(lf)</i>	30 (294)	<0.0001 to <i>slo-1(lf)</i>
<i>unc-103(0); egl-2(0) slo-1(lf)</i>	79 (42)	
<i>unc-103(sy557)</i>	75 (40)	0.7 to <i>slo-1(lf)</i>

is determined by isolating virgin larval males from hermaphrodites. The males are allowed to mature to adulthood, and the phenotype is scored 18 to 24 hr later. A male that displays at least partial protraction of one spicule is designated as displaying the Prc phenotype. We previously identified two members of the *ether-a-go-go* (EAG) K⁺ channel family, EAG and *ether-a-go-go*-related gene (ERG), that regulate spicule protraction (Garcia and Sternberg 2003; LeBoeuf *et al.* 2007). In *C. elegans*, EAG is encoded by *egl-2* and in the male spicule protraction circuit is expressed in sex muscles (Weinshenker *et al.* 1999); males with a null mutation in the K⁺ channel display no obvious mating defects (0% of the males display the Prc phenotype) (Table 1) (LeBoeuf *et al.* 2007). ERG is encoded by *unc-103* and is expressed in nearly all muscles and neurons (Reiner *et al.* 2006). In contrast to *egl-2* mutants, males with a null mutation in *unc-103* display spicule protraction defects (41% of the males display the Prc phenotype) (Table 1) (Garcia and Sternberg 2003). Mutating both *unc-103* and *egl-2* voltage-dependent K⁺ channel genes induce the Prc phenotype in 83% of males (Table 1) (LeBoeuf *et al.* 2011). While the penetrance of the Prc phenotype is much higher in the double mutant males than either single mutant, not all males in a population lacking both K⁺ channels displayed the Prc phenotype. This led us to ask whether other K⁺ channels are involved in maintaining cell excitability.

One likely candidate is the voltage- and calcium-activated BK channel *slo-1*, which is broadly expressed in neurons and muscles (Wang *et al.* 2001; Kim *et al.* 2009). *slo-1* loss-of-function mutants exhibit many phenotypes, including impaired movement caused by defective neurotransmitter release, alcohol resistance, and muscle degeneration (Wang *et al.* 2001; Davies *et al.* 2003; Carre-Pierrat *et al.* 2006; Liu *et al.* 2007a). We found that 70% of males with an early nonsense mutation in *slo-1* [allele *js379*, hereafter referred to as *slo-1(lf)*; Wang *et al.* 2001] spontaneously protract their spicules in the absence of mating cues (Table 1), demonstrating that like EAG and ERG-like K⁺ channels, the BK channel also controls the excitability of male sex circuit components.

slo-1 has been shown in *C. elegans* and other systems to interact with calcium/calmodulin-dependent protein kinase II (CaMKII) (Hawasli *et al.* 2004; Liu *et al.* 2006, 2007a; LeBoeuf *et al.* 2007). *unc-43* encodes CaMKII in *C. elegans*

and regulates movement, defecation, and egg laying in addition to its role in male mating (Reiner *et al.* 1999). We previously reported that loss-of-function mutations in CaMKII cause *C. elegans* males to display the Prc phenotype. The *sy574* allele in *unc-43* changes a glycine to a glutamate in the putative substrate binding site of CaMKII. A total of 38% of *unc-43(sy574)* males displayed the Prc phenotype but exhibit no other obvious defects (Table 1) (LeBoeuf *et al.* 2007). To determine whether the frequency of the *unc-43(sy574)*-mediated Prc phenotype was related to reduced SLO-1 function, we created a double mutant containing *slo-1(lf)* and *unc-43(sy574)* to ask whether the K⁺ channel genetically acts downstream of CaMKII. In contrast to the single mutant males, we found that the incidence of the Prc phenotype in double mutant males was 96% [P value <0.0001 to *slo-1(lf)*] (Table 1). This synergistic effect suggests that while *slo-1* might be a target of *unc-43* in other behavioral contexts, it is not the only CaMKII target in the male mating circuit.

Specific isoforms of sex muscle-expressed BK channel/*slo-1* can reduce abnormal protraction

Since *slo-1* is involved in regulating the timing of male sex muscle contractions, we asked where it functions in the mating circuit. We obtained a construct (P_{slo-1}::*slo-1*(A2;B0;C1;D0)::GFP) containing the *slo-1* isoform previously used to restore function in hermaphrodites with movement defects (Wang *et al.* 2001). *slo-1* has four identified splice regions labeled A–D, therefore isoform A2;B0;C1;D0 includes the exons at A2 and C1 (Figure 1) (Johnson *et al.* 2011). Splice sites A–C were previously identified (Wang *et al.* 2001), whereas we identified D during cloning of full-length *slo-1* cDNAs (Figure 1C) (Materials and Methods). We introduced P_{slo-1}::*slo-1*(A2;B0;C1;D0)::GFP into *slo-1(lf)* worms and discovered that spicule protraction was reduced from 61 to 38% (*P* = 0.02) (Table 2). We then generated tissue-specific constructs to express *slo-1*(A2;B0;C1;D0) in neurons (P_{aex-3}), body-wall muscles (P_{acr-8}), and sex muscles (P_{unc-103E}) (Reiner *et al.* 2006; LeBoeuf *et al.* 2007). *slo-1*(A2;B0;C1;D0) expression in neurons and body-wall muscles did not reduce the Prc phenotype, whereas expression in the sex muscles reduced the incidence of spontaneous protraction

Figure 1 *slo-1* gene structure and splice variants used in this study. (A) Boxes indicate relative exon sizes and location. A letter is used to designate an alternatively spliced region; a number designates an alternatively spliced site. A letter followed by 0 indicates where splicing occurs when the alternative exon is not included. For splice region A, either the exon at A1 or A2 is included in all isoforms. For splice regions B–D, the exons are included in some isoforms but not in others. The shaded box indicates the exon newly described in this work; when present in an isoform, it is spliced between previously described exons 2 and 3. The open boxes indicate the specific exons differentially spliced in the three isoforms analyzed in this study. (B) Comparison of amino acid sequences for exons 9 (splice site A1) and 10 (splice site A2). The open boxes indicate amino acids that differ between the two exons. (C) The amino acid sequence for the newly described exon at splice site D1. (D) The sequence change for the mutant *slo-1(rg432)* located in an intron. The top letter is the wild-type sequence, while the bottom letter is the mutant sequence.

to 23% [$P = 0.0005$ to *slo-1(lf)*] (Table 2). Thus, *slo-1* is partially regulating spicule protraction in the sex muscles.

Though *slo-1(A2;B0;C1;D0)* significantly reduces the instance of spontaneous protraction, a large percentage of the males (38%) expressing this isoform still displayed the mu-

tant phenotype (Table 2). *slo-1(A2;B0;C1;D0)* is one isoform of at least 14 that exist in *C. elegans* (Wang *et al.* 2001; Johnson *et al.* 2011). We asked whether another isoform is capable of complete rescue of the mutant phenotype. We tested two additional isoforms of *slo-1*, *slo-1(A2;B0;C0;*

Table 2 Transgenic rescue of mutant BK/*slo-1* and ERG/*unc-103*-induced spicule protraction

Genotype	Transgene	Tissue expression	% spicule protracted (n)	P value, Fisher's exact test
Wild type			12 (42)	
<i>slo-1(lf)</i>			61 (83)	
<i>slo-1(lf)</i>	<i>rgEx403[P_{slo-1}:slo-1(A2;B0;C1;D0)]</i>	Most neurons and muscles	38 (40)	0.02 to <i>slo-1(lf)</i>
<i>slo-1(lf)</i>	<i>rgEx401[P_{aex-3}:slo-1(A2;B0;C1;D0)]</i>	All neurons	61 (31)	
<i>slo-1(lf)</i>	<i>rgEx405[P_{acr-8}:slo-1(A2;B0;C1;D0)]</i>	Body-wall muscle	75 (36)	
<i>slo-1(lf)</i>	<i>rgEx404[P_{unc-103E}:slo-1(A2;B0;C1;D0)]</i>	Sex muscle	23 (30)	0.0005 to <i>slo-1(lf)</i>
<i>slo-1(lf)</i>	<i>rgEx520[P_{slo-1}:slo-1(A2;B0;C0;D0)]</i>	Most neurons and muscles	5 (42)	<0.0001 to <i>slo-1(lf)</i>
<i>slo-1(lf)</i>	<i>rgEx456[P_{slo-1}:slo-1(A1;B0;C0;D0)]</i>	Most neurons and muscles	0 (35)	<0.0001 to <i>slo-1(lf)</i>
<i>slo-1(lf)</i>	<i>rgEx469[P_{unc-103E}:slo-1(A1;B0;C0;D0)]</i>	Sex muscle	9 (32)	<0.0001 to <i>slo-1(lf)</i>
<i>unc-103(0)</i> ^a			26 (53)	
<i>unc-103(sy557)</i>			75 (40)	<0.0001 to <i>unc-103(0)</i>
<i>unc-103(0)</i>	<i>rgEx86[P_{unc-103E}:unc-103(sy557A)]</i>	Sex muscle	22 (45)	
<i>unc-103(0)</i>	<i>rgEx477[P_{unc-103F}:unc-103(sy557A)]</i>	Sex neurons	32 (38)	
<i>unc-103(0)</i>	<i>rgEx478[P_{unc-103E}:unc-103(sy557B)]</i>	Sex muscle	16 (37)	
<i>unc-103(0)</i>	<i>rgEx87[P_{unc-103F}:unc-103(sy557B)]</i>	Sex neurons	23 (40)	
<i>unc-103(0)</i>	<i>rgEx479[P_{unc-103E}:unc-103(sy557A)+P_{unc-103F}:unc-103(sy557A)]</i>	Sex muscle and sex neurons	15 (33)	
<i>unc-103(0)</i>	<i>rgEx192[P_{unc-103E}:unc-103(sy557B)+P_{unc-103F}:unc-103(sy557B)]</i>	Sex muscle and sex neurons	62 (52)	0.008 to <i>unc-103(0)</i>
<i>egl-2(0) slo-1(lf)</i>			33 (55)	
<i>egl-2(0) slo-1(lf)</i>	<i>rgEx244[P_{unc-103F}:unc-103(sy557B)]</i>	Sex neurons	32 (47)	
<i>egl-2(0) slo-1(lf)</i>	<i>rgEx242[P_{unc-103E}:unc-103(sy557B)]</i>	Sex muscle	72 (89)	<0.0001 to <i>slo-1(lf) egl-2(0)</i>
<i>egl-2(0) slo-1(lf)</i>	<i>rgEx245[P_{unc-103E}:unc-103(sy557B)+P_{unc-103F}:unc-103(sy557B)]</i>	Sex muscle and sex neurons	81 (32)	<0.0001 to <i>slo-1(lf) egl-2(0)</i>

^a *unc-103(0)* animals contain *pha-1(e2123)*.

D0) and *slo-1(A1;B0;C0;D0)*. *slo-1(A2;B0;C0;D0)* does not have the exon at splice site C1 and was first reported in Wang *et al.* (2001). *slo-1(A1;B0;C0;D0)* exchanges one conserved hydrophobic region 38 amino acids in length in the C terminus with another 38-amino-acid sequence of high homology (Figure 1, A and B). When expressed from the *slo-1* promoter, both *slo-1(A2;B0;C0;D0)* and *slo-1(A1;B0;C0;D0)* reduced spicule protraction (from 61% to 5 and 0%, respectively) (Table 2). Additionally, tissue-specific expression of *slo-1(A1;B0;C0;D0)* in the sex muscles via the *unc-103E* promoter resulted in a 9% instance of the Prc phenotype [*P* value <0.0001, compared to *slo-1(lf)*] (Table 2). In conclusion, the exon located at C1 reduces *slo-1*'s ability to inhibit premature spicule protraction and demonstrates that isoform specificity does play a role in restoring male mating behavior.

BK channel/*slo-1(lf)*-induced sex muscle spasms can be modified by EAG family *K⁺* channels

Since mutations in BK/*slo-1*, ERG-like/*unc-103*, and EAG/*egl-2* differentially regulate the excitability of the sex muscles, we asked whether simultaneous loss of multiple *K⁺* channels would have an additive or even a synergistic effect on male mating circuit excitability. We made double mutants between *slo-1(lf)* and strains that carry large deletions of *unc-103* and *egl-2* [hereafter referred to as *unc-103(0)* and *egl-2(0)*] (Park and Horvitz 1986; Garcia and Sternberg 2003; LeBoeuf *et al.* 2007). Contrary to our initial expectations, both *unc-103(0); slo-1(lf)* and *egl-2(0) slo-1(lf)* double mutants displayed a decrease in the instance of the Prc phenotype [*unc-103(0); slo-1(lf)* and *egl-2(0) slo-1(lf)* dropped the frequency of the Prc phenotype to 51 and 30%, respectively, lower than the *slo-1(lf)*-induced 70% protraction] (Table 1). Thus, removing an ERG-like or an EAG *K⁺* channel along with a BK channel from the male mating circuit lowers rather than increases sex muscle excitability. Males lacking all three *K⁺* channels, an *unc-103(0); egl-2(0) slo-1(lf)* triple mutant, protracted their spicules 79% of the time, a frequency similar to the *slo-1(lf)* single mutant and the *unc-103(0); egl-2(0)* double mutant (Table 1). This suggests that when either *egl-2* or *unc-103* is removed from the *slo-1* background, some aspect (transcriptional, translational, or post-translational) of the remaining *K⁺* channels might be upregulated to compensate for the loss.

The nonadditive effect of combining the *slo-1(lf)* mutation with either *unc-103(0)* or *egl-2(0)* suggests the existence of mechanisms that sense the level of cell excitability and adjusts extant *K⁺* channels to partially compensate for missing or defective *K⁺* channels. To address this hypothesis, we utilized an allele described in an earlier report, a neomorphic loss-of-function allele (*sy557*) in the *unc-103* ERG-like *K⁺* channel gene. *unc-103(sy557)* contains two point mutations: *sy557A*, a H165N change in the linker region between transmembrane domains two and three, and *sy557B*, a W244R change in transmembrane domain five (Garcia and Sternberg 2003). *unc-103(sy557)* males displayed a Prc frequency of

75%, significantly higher than males completely lacking *unc-103* (*P* = 0.0005, compared to *unc-103(0)* at 41%) (Table 1), suggesting that the mutant *K⁺* channel gene adversely affects compensating molecules. Since the frequency of *unc-103(sy557)* males displaying the Prc phenotype was similar to *unc-103(0); egl-2(0)* double and *slo-1(lf)* single mutant males, we hypothesized that the *sy557*-encoded mutation(s) interferes with the compensating properties of EGL-2 and/or SLO-1.

To address which *sy557* mutation confers the neomorphic dominant negative properties to UNC-103, we mutated a construct containing *unc-103* genomic DNA to encode either the *sy557A* H165N or *sy557B* W244R change (Garcia and Sternberg 2003). We then expressed the mutated *unc-103* sequences in *unc-103(0)* male sex muscles using promoter *unc-103E* [*P_{unc-103E}:unc-103(sy557A or B)*] or sex neurons using promoter *unc-103F* [*P_{unc-103F}:unc-103(sy557A or B)*] to determine whether transgenic mutated versions of *unc-103* can interfere with compensating mechanisms and increase the Prc frequency (Reiner *et al.* 2006). Neither UNC-103(H165N) nor UNC-103(W244R) expressed individually in the sex muscles or neurons increased the rate of spontaneous spicule protraction (Table 2). However, expressing UNC-103(W244R), but not UNC-103(H165N), in both sex muscles and neurons significantly increased protraction from 26 to 62% (*P* = 0.008, Fisher's exact test) (Table 2). Thus, disrupting *unc-103* via a W244R change in transmembrane domain five can interfere with additional neural and muscle-compensating functions to consequently increase the instance of unregulated spicule protraction.

To determine whether the compensating functions that UNC-103(W244R) interferes with are related to EGL-2 and/or SLO-1 activity, we transgenically introduced the neomorphic dominant negative UNC-103(W244R) into *egl-2(0) slo-1(lf)* double mutant males. We then asked whether the frequency of the Prc phenotype is similar to *unc-103(0); egl-2(0) slo-1(lf)* triple mutants or higher. We hypothesized that if there is a genetic interaction between wild-type *slo-1*, *egl-2* and *unc-103*, then UNC-103(W244R) would interfere with wild-type *slo-1* and/or *egl-2* activity and the males would display a Prc frequency of ~70–80%. A testable prediction of this hypothesis is that if UNC-103(W244R) is transgenically introduced into *egl-2(0) slo-1(lf)* double mutants, then UNC-103(W244R) should only interfere with wild-type *unc-103*, since the other two *K⁺* channels are already mutant; consequently the transgenic males should also display a Prc frequency of ~70–80%. If the hypothesis is incorrect, and UNC-103(W244R) interferes with something other than *egl-2* and *slo-1* function, the transgenic males should display a Prc frequency greater than the *unc-103(0); egl-2(0) slo-1(lf)* triple mutant. As previously stated, *egl-2(0) slo-1(lf)* males were 30% Prc, whereas *slo-1(lf)* males were 70% Prc (Table 1); this decrease in the Prc frequency in the double mutant could be due to compensation by *unc-103*. We expressed UNC-103(W244R) in male sex muscles and neurons, individually and together, in *egl-2(0) slo-1(lf)* males. UNC-103(W244R) expressed in neurons of *egl-2(0)*

slo-1(lf) males had no effect on spicule protraction (Table 2). However, UNC-103(W224R) expressed in the sex muscles was sufficient to return spontaneous spicule protraction to levels seen in *slo-1(lf)* mutants (72% of the males displayed the Prc phenotype) (Table 2). Expressing UNC-103(W224R) in both sex muscles and neurons was not significantly different from muscles alone (81% of the males displayed the Prc phenotype) (Table 2). In conclusion, UNC-103(W224R) appears to interfere with only *unc-103* in an *egl-2(0) slo-1(lf)* background. Since the instance of spontaneous spicule protraction was similar to that seen in *unc-103(sy557)* and *unc-103(0); egl-2(0) slo-1(lf)* mutant males, *unc-103(sy557)* likely also interferes with *egl-2* and *slo-1*. These data are consistent with the idea that there is a genetic interaction between *slo-1*, *egl-2*, and *unc-103*.

BK/*slo-1* expression is increased in the absence of *ERG/unc-103* but not *EAG/egl-2*

The genetic experiments discussed in the preceding sections suggested that BK channel/*slo-1* function or expression is possibly upregulated in the absence of EAG family K⁺ channels *unc-103* and *egl-2*. To ask whether *slo-1* expression in the male sex muscles might be changed in mutant backgrounds, we transgenically expressed dsRed1-E5 from the *slo-1* promoter as a proxy of promoter activity. dsRed1-E5 shifts its emission spectra from green to red over time, and we previously used it to differentiate fluorescent proteins that were newly expressed from older accumulated proteins (Terskikh *et al.* 2000; LeBoeuf *et al.* 2011). We expressed integrated P_{*slo-1*}:dsRed1-E5 in wild type, *unc-103(0)*, *unc-103(sy557)*, and *egl-2(0)* males. We then asked whether removing the K⁺ channels affected marker gene expression in the tails over the course of L4 development and 2 days of adulthood. In L4 wild-type males, we measured the marker gene expression in the male anal depressor muscle, an accessory to the spicule protractor muscles. Since the anal depressor muscle differentiates early in the embryo (Sulston *et al.* 1983), dsRed1-E5 expression was higher in the red channel than the green (Figure 2, A and D, *P* value <0.05 for red vs. green for all genotypes, Mann–Whitney *t*-test). However, the fluorescence intensity difference in the L4 anal depressor between the green and red channels changed when *unc-103(0)* was introduced into the genetic background (Figure 2A). P_{*slo-1*}:dsRed1-E5 expression in *unc-103(0)* male tails was increased in both channels relative to the wild type (Figure 2A). This suggests that in the absence of *unc-103*, the transgenic and possibly the endogenous *slo-1* promoter is more active and indicates that more *slo-1* gene product is made in an attempt to compensate. In contrast, P_{*slo-1*}:dsRed1-E5 expression was unchanged compared to wild type in *egl-2(0)* male tails (Figure 2A). P_{*slo-1*}:dsRed1-E5 expression was also unchanged in *unc-103(sy557)* males (Figure 2A), suggesting that unlike the *unc-103(0)* allele, the presence of this non-functioning UNC-103 protein does not promote an increase in *slo-1* expression.

In day 1 and day 2 wild-type adult males, we measured marker gene expression in the fully developed male protractor muscles and the anal depressor accessory muscle. P_{*slo-1*}:dsRed1-E5 expression in the green channel was higher than in the red channel, due to the differentiation of the sex muscles (Figure 2, B and E, *P* value <0.05, Mann–Whitney *t*-test). There was no difference between the green and red channels in males lacking EGL-2 or UNC-103 K⁺ channels, though expression in the red channel was significantly higher than the green channel in *unc-103(sy557)* males (Figure 2B, *P* value <0.05, Mann–Whitney *t*-test). However relative to wild-type animals, there was a significant increase in marker gene expression when *unc-103* was deleted (Figure 2B). In contrast, marker gene expression in *egl-2(0)* males was not significantly different from the wild type (Figure 2B). Therefore, similar to L4 males, the deletion of *unc-103* caused an increase in transgenic and possibly endogenous *slo-1* promoter activity but the loss of *egl-2* had no effect. P_{*slo-1*}:dsRed1-E5 expression in *unc-103(sy557)* males was significantly higher in the red but not the green channel, likely due to the large variability in expression seen among these males. Individual males could have differing responses to the loss of excitability regulation induced by the *unc-103(sy557)* allele.

In day 2 wild-type adult males, expression in the red channel was much higher than in the green channel, due to the accumulation of dsRed1-E5 for 2 days (Figure 2, C and F). Similar to L4 and 1-day-old adult males, P_{*slo-1*}:dsRed1-E5 expression was higher in *unc-103(0)* mutants but unaffected in *egl-2(0)* mutants (Figure 2C). This is further evidence that a higher rate of *slo-1* expression is maintained in *unc-103(0)* males as they age. In contrast, P_{*slo-1*}:dsRed1-E5 expression is lower in 2-day-old *unc-103(sy557)* males (Figure 2C), a reversal of the slight increases in expression in the younger adults. These data suggest that while *slo-1* levels might be similar to wild type in young *unc-103(sy557)* adult animals, this rate is not maintained as they age.

Food deprivation can attenuate BK channel/*slo-1(lf)*-induced spicule protraction through *EAG K⁺ channel/egl-2*

In previous work, we demonstrated that starvation can suppress the excitability of the mating circuit in ERG-like K⁺ channel/*unc-103* mutants through enhanced compensation by *EAG/egl-2* function (Gruninger *et al.* 2006; LeBoeuf *et al.* 2007, 2011). Since we established that there is a genetic interaction between *slo-1*, *egl-2*, and *unc-103*, we asked whether food-deprived BK channel/*slo-1* mutant males, like *unc-103(0)* animals, had a reduced frequency of the Prc phenotype via enhanced *egl-2* compensation. *C. elegans* matures through four larval stages (L1–L4) to reach adulthood; the male sex muscles differentiate in the last stages of L4. We starved males by picking them at the late L4 stage and placed them on NGM plates lacking their food source, *E. coli* OP50. Males develop normally under these starvation conditions (Gruninger *et al.* 2006). After 18–22 hr, we scored whether the starved males displayed the spontaneous spicule

Figure 2 *slo-1* promoter expression increases in an *unc-103(0)* mutant background. (A–C) Mean gray level of DsRed-E5 fluorescent protein expression in the male tail. DsRed-E5 initially expresses in the green channel and then shifts its emission spectra to the red channel. x-axis indicates the genotype of the male and fluorescent channel measured, while the y-axis indicates the amount of fluorescence (mean gray level) measured in the male tail (A.U., arbitrary units). Error bars represent standard deviation. **P* value <0.05, ***P* value <0.005, ****P* value <0.0001; Mann–Whitney *t*-test. (A) L4 stage males. Wild type *n* = 29, *unc-103(0)* *n* = 26, *egl-2(0)* *n* = 38, and *unc-103(sy557)* *n* = 22. (B) One-day-old males. Wild type *n* = 34, *unc-103(0)* *n* = 29, *egl-2(0)* *n* = 21, and *unc-103(sy557)* *n* = 23. (C) Two-day-old males. Wild type *n* = 33, *unc-103(0)* *n* = 23, *egl-2(0)* *n* = 21, and *unc-103(sy557)* *n* = 20. (D–F) Fluorescent images of wild-type male tails. (Left) Green channel. (Right) Red channel. Bars, 20 μ m. Open boxes indicate area used to determine mean gray level. The male is oriented so that the top of the image is anterior and the left is ventral. (D) Mid-L4 male tail (the stage in L4 when the male tail spike completes its retraction). (E) One-day-old adult male tail. (F) Two-day-old adult male tail.

protraction phenotype. We found that 31% of food-deprived *slo-1(lf)* males displayed the Prc phenotype, significantly lower than *slo-1(lf)* males on food (70% displayed the Prc phenotype, *P* value <0.0001) (Table 3). Thus, like *unc-103* mutants, starvation is able to partially suppress *slo-1(lf)*–induced protraction.

We then asked whether starvation was able to inhibit protraction in males lacking both *slo-1* and *egl-2*. We found that depriving *egl-2(0) slo-1(lf)* males of food had no effect on the instance of Prc (31 vs. 30%, *P* = 0.7) (Table 3), consistent

with the idea that *egl-2* mediates the effects of starvation on the excitability of the mating circuit. To support the idea that in the absence of food, enhanced *egl-2* compensation can reduce *slo-1(lf)*–induced excitability defects, we expressed an *egl-2* gain-of-function allele in the male sex muscles and asked whether the frequency of *slo-1(lf)*–induced spicule protraction can be further suppressed by starvation.

The *egl-2(gf)* allele induces egg retention in hermaphrodites as well as defecation and chemotaxis defects (Reiner *et al.* 1995; Weinshenker *et al.* 1995; Weinshenker *et al.*

Table 3 Effects of starvation on K⁺ channel mutation-induced spicule protraction

Genotype	% spicule protracted males (n)		P value
	Fed	Starved	Fisher's exact test ^a
Wild type	3 (36)	0 (38)	
<i>slo-1(lf)</i>	70 (201)	31 (178)	<0.0001
<i>egl-2(0)</i>	0 (40)	0 (35)	
<i>egl-2(0) slo-1(lf)</i>	30 (294)	31 (295)	0.7
<i>egl-2(gf)</i>	8 (39)	0 (33)	
<i>egl-2(gf) slo-1(lf)</i>	91 (138)	53 (32)	<0.05
<i>egl-2(0) slo-1(lf);</i> <i>rgEx253[P_{unc-103E}:egl-2(gf)]</i>	66 (32)	7 (30)	<0.05
<i>slo-1(rg432)</i>	6 (36)	0 (33)	
<i>unc-103(0)</i>	41 (80)	0 (47)	<0.0001
<i>unc-103(0); slo-1(rg432)</i>	51 (40)	9 (33)	<0.0001
<i>egl-2(0) slo-1(rg432)</i>	3 (34)	3 (35)	
<i>unc-103(0); egl-2(0)</i>	83 (46)	34 (99)	<0.0001
<i>unc-103(0); egl-2(0) slo-1(rg432)</i>	46 (35)	13 (70)	<0.005

^a Comparing fed vs. starved of the same genotype.

1999). In the male mating circuit, *egl-2(gf)* suppresses muscle excitability when activated by starvation (LeBoeuf *et al.* 2011). We made a double mutant containing both *slo-1(lf)* and *egl-2(gf)* and found that the double mutant males were extremely constipated and in 91% of the males, the proctodeum, including the spicules, hemorrhaged from the cloacal opening.

slo-1 has a broad expression pattern in neurons and muscles outside the male mating circuit, whereas *egl-2* is expressed in a few head neurons and intestinal muscles (Weinshenker *et al.* 1999; Wang *et al.* 2001). The *slo-1(lf)* and *egl-2(gf)* alleles appear to cause a synthetic effect resulting in chronic constipation and hemorrhaging. To circumvent this problem, we expressed *egl-2(gf)* cDNA only in the sex muscles of *egl-2(0) slo-1(lf)* males using the *unc-103E* promoter. A total of 66% of *egl-2(0) slo-1(lf); rgEx253 [P_{unc-103E}:egl-2(gf)]* males display abnormal spicule protraction, significantly lower than *egl-2(gf) slo-1(lf)* males and similar to *slo-1(lf)* males (91 and 70%, *P* values <0.005 and =0.7, respectively) (Table 3). Starvation reduced spicule protraction in *egl-2(0) slo-1(lf); rgEx253 [P_{unc-103E}:egl-2(gf)]* males to 7% (*P* value <0.05 to fed) (Table 3). Thus, *egl-2* compensation can be stimulated by starvation to reduce sex muscle excitability, and this increase in *egl-2* activity can reduce *slo-1(lf)*-induced muscle spasms.

***slo-1(rg432)* encodes an intronic point mutation that reduces sex muscle excitability**

As we have previously stated, *unc-103(0); egl-2(0)* double mutant males display spontaneously protracted spicules at a frequency of 83%. In the course of working with the double mutant, we realized that a spontaneous genetic modifier (*rg432*) unknowingly got crossed into the strain. The modifier reduced the frequency of the *unc-103(0); egl-2(0)* double mutant Prc phenotype to 46% [*P* value <0.005, compared to *unc-103(0); egl-2(0)*] (Table 3). In addition, we discovered that starvation strongly suppressed the instance of the Prc phenotype for *unc-103(0); egl-2(0)* animals that contained the *rg432* modifier [*P* value <0.005, com-

pared to *unc-103(0); egl-2(0)*] (Table 3). We roughly mapped the genetic modifier (through conventional crosses, data not shown), and not surprisingly, it mapped close to *slo-1*. We sequenced *slo-1* from the *unc-103(0); egl-2(0) rg432* line and found an A-to-T change in an intron between exons 11 and 12 (Figure 1D). This mutation was not present in the normal *unc-103(0); egl-2(0)* line. When unlinked to *egl-2(0)* and *unc-103(0)*, animals harboring the *slo-1(rg432)* allele appeared behaviorally wild type under fed or starved conditions (Table 3). In double mutant combinations, *slo-1(rg432)* had no effect on the frequency of the Prc phenotype of *unc-103(0)* or *egl-2(0)* males (Table 3). Since *unc-103(0); egl-2(0) slo-1(rg432)* triple mutant males displayed a similar Prc phenotype to *unc-103(0)* single mutant males (46 vs. 41%, *P* = 0.7, Fisher's exact test) (Table 3), it was possible that the *slo-1(rg432)* allele facilitated increased SLO-1 compensation in the absence of *egl-2*.

It has recently been reported that a point mutation in a *slo-1* intron can affect alternative splicing (Glauser *et al.* 2011). Since the *slo-1(rg432)* allele is located in an intron, we asked whether alternative splicing was affected. The *slo-1* genetic locus contains four alternatively spliced regions, labeled A–D, and the *rg432* mutation does not map to any of them (Figure 1D). We designed primers to distinguish between the differentially spliced exons and performed quantitative RT-PCR on cDNA obtained from wild-type and *slo-1(rg432)* nonstarved adult hermaphrodites or males. Total *slo-1* expression was greatly increased from males to hermaphrodites, likely due to the difference in the number of excitable cells (sex-specific muscles and neurons) between the two sexes, and this was true for all isoforms (Figure 3, A and B). Similar to wild type, *slo-1(rg432)* males showed increased expression when compared to mutant hermaphrodites, and this effect was not isoform specific (Figure 3, A and B). *slo-1* transcript levels were decreased in *slo-1(rg432)* hermaphrodites, with some splice sites being significantly affected while others were not (Figure 3, A and B). Importantly, *slo-1* mRNA levels were significantly lower in

Figure 3B

<i>slo-1</i> splice variant	Normalized Fold Expression			
	Wild type		<i>slo-1(rg432)</i>	
	Herm	Male	Herm	Male
All	1.00	2.07 ^a	0.57 ^b	0.86 ^{d,e}
A1	1.00	2.43 ^a	0.63	1.15 ^{d,e}
A2	1.00	3.60 ^a	1.36	2.00 ^{c,d,e}
B0	1.00	2.97 ^a	0.82	1.13 ^{d,e}
B1	1.00	3.72 ^a	0.47 ^b	1.11 ^{d,e}
C0	1.00	3.04 ^a	0.78	1.25 ^{d,e}
C1	1.00	3.15 ^a	0.48 ^b	0.94 ^{d,e}
D0	1.00	3.37 ^a	1.02	1.71 ^{c,d,e}
D1	1.00	3.11 ^a	0.65	1.01 ^{d,e}

Figure 3D

gene	Normalized Fold Expression			
	Wild type		<i>slo-1(rg432)</i>	
	Herm	Male	Herm	Male
<i>acr-18</i>	1.00	1.26	0.62 ^b	0.68 ^d
<i>gar-3</i>	1.00	2.41 ^a	0.50 ^b	1.26 ^{d,e}
<i>unc-29</i>	1.00	3.72 ^a	0.48 ^b	1.55 ^{c,d,e}
<i>unc-43</i>	1.00	2.66 ^a	1.14	1.16 ^{c,d}
<i>cat-2</i>	1.00	0.89	1.73 ^b	0.65 ^{c,e}
<i>let-363</i>	1.00	2.24 ^a	1.71 ^b	0.73 ^{c,d,e}
<i>sir-2.1</i>	1.00	1.48 ^a	2.04 ^b	0.89 ^{d,e}
<i>cdc-42</i>	1.00	0.85	2.30 ^b	0.81 ^e
<i>pmp-3</i>	1.00	1.17	3.10 ^b	0.77 ^{c,d,e}

"b" compares wild-type hermaphrodites to *slo-1(rg432)* hermaphrodites; "c" compares wild-type hermaphrodites to *slo-1(rg432)* males; "d" compares wild-type males to *slo-1(rg432)* males, and "e" compares *slo-1(rg432)* hermaphrodites to *slo-1(rg432)* males. Herm, hermaphrodite.

slo-1(rg432) males, resulting in a much more pronounced effect on *slo-1* expression levels by the mutant allele in males vs. hermaphrodites (Figure 3, A and B). Thus, the *slo-1(rg432)* allele results in a decrease in *slo-1* transcript levels, which is most profound in males and is not isoform specific in adults.

Stable K⁺ channel transcription is dependent on BK channel/*slo-1* and EAG K⁺ channel/*egl-2*

Our experiments discussed so far suggest that K⁺ channels in the male sex muscles can be upregulated to compensate for one another to control muscle excitability. The dsRed1-E5 marker gene analysis indicated that increases in gene expression via mRNA synthesis or stability could be one method of upregulation. To determine whether K⁺ channel compensation via mRNA synthesis or stability is more general and not just limited to the male spicule muscles, we measured global relative changes in K⁺ channel mRNA abundance in wild type and *unc-103*, *egl-2*, and *slo-1* mutants. We isolated poly(A) mRNA from well-fed mixed gender and mixed staged whole worm extracts and performed quantitative RT-PCR experiments.

First, we measured global *unc-103* and *slo-1* transcript levels in *egl-2(0)* worms and found that K⁺ channel transcript levels were significantly decreased (Figure 4, A, C, and D). Thus, the relative normalcy of *egl-2(0)* worms cannot be explained by an increase in *unc-103* and *slo-1* transcript

levels. Likewise, *unc-103(sy557)* worms displayed unaffected (*egl-2*) or decreased (*slo-1* and *unc-103*) levels of transcript (Figure 4). However, *egl-2* and *slo-1* transcript levels were increased in *unc-103(0)* worms (Figure 4, B–D), indicating that gene expression is a possible mechanism of attempted compensation in these worms. Additionally, *unc-103(sy557)* males displayed a higher instance of the Prc phenotype than *unc-103(0)* males, a difference that could be due in part to the lack of K⁺ channel upregulation in *unc-103(sy557)* mutants.

We next measured K⁺ channel transcript levels in *slo-1(lf)* and *slo-1(rg432)* worms. While *slo-1* transcript levels were unaffected in *slo-1(rg432)* mutants, *egl-2* and *unc-103* levels were decreased (Figure 4). The *slo-1(rg432)* allele promotes regulation of male sex muscle excitability, but it is not due to an increase in transcription or mRNA stability. *slo-1(lf)* mutants display a high level of the Prc phenotype, so it was unsurprising to see that *unc-103* and *egl-2* levels were significantly decreased (Figure 4). Also as expected, the levels were significantly lower than wild type in the *slo-1(lf)* mutant containing an early nonsense mutation (Figure 4, C and D), likely as a result of nonsense-mediated mRNA decay (Hodgkin *et al.* 1989; Maquat 2004; Longman *et al.* 2007). The primers used to detect *slo-1* transcripts start at amino acid F641, considerably downstream from the Q251stop mutation in the loss-of-function mutant. There is little to suggest extensive read-through of the stop mutation,

Figure 3 Variations in male and hermaphrodite gene transcript levels. qRT-PCR was performed on populations of young adult hermaphrodites and young adult males in wild type and *slo-1(rg432)* backgrounds. Error bars represent standard deviation. (A and B) Transcript levels of *slo-1* splice variants. (C and D) Transcript levels of various genes. (A) x-axis indicates the *slo-1* splice site tested, while the y-axis indicates the normalized fold expression. (B) x-axis indicates the gene tested, while the y-axis indicates the normalized fold expression. (A and C) Dark blue bars, wild-type hermaphrodites; light blue bars, wild-type males; red bars, *slo-1(rg432)* hermaphrodites; pink bars, *slo-1(rg432)* males. (A and C) Data here are reformatted in table form in B and D for ease of comparison. (B and D) Letters next to numbers indicate a P value of <0.05, unpaired t-test with Welch's correction. Footnote symbols are as follows: "a" compares wild-type males to wild-type hermaphrodites; "b" compares wild-type hermaphrodites to *slo-1(rg432)* hermaphrodites; "c" compares wild-type hermaphrodites to *slo-1(rg432)* males; "d" compares wild-type males to *slo-1(rg432)* males, and "e" compares *slo-1(rg432)* hermaphrodites to *slo-1(rg432)* males. Herm, hermaphrodite.

Figure 4 *slo-1*, *egl-2*, and *unc-103* K⁺ channel transcript levels. qRT-PCR performed on wild-type and K⁺ channel mutants. (A–C) **P* < 0.05, ***P* < 0.005, ****P* < 0.0005, unpaired t-test, mutant compared to wild type. Error bars represent standard deviation. x-axis indicates the genotype tested, y-axis is the level of normalized gene expression. (A) ERG/*unc-103* expression. (B) EAG/*egl-2* expression. (C) BK/*slo-1* expression. (D) The data in A–C are reformatted as a table for ease of comparison. (**P* value < 0.05, statistically significant difference from wild type; unpaired t-test).

due to the severe defects shown by *slo-1(lf)* animals, indicating functional SLO-1 K⁺ channels are greatly reduced if not abolished (Wang *et al.* 2001; Carre-Pierrat *et al.* 2006).

Since the frequency of the Prc phenotype was significantly decreased in *unc-103(0); egl-2(0) slo-1(rg432)* as compared to *unc-103(0); egl-2(0)* males (Table 3), we asked how global *slo-1* mRNA levels were affected in the triple and double mutants relative to wild type and the single mutants. In *unc-103(0); egl-2(0)* animals, the global *slo-1* transcript abundance were similar to the wild type. This could be an additive consequence of the *unc-103(0)* mutation increasing *slo-1* transcript levels, balanced with the effect of *egl-2(0)* decreasing them. Unexpectedly, *slo-1* mRNA levels were drastically lower in *unc-103(0); egl-2(0) slo-1(rg432)* mutants relative to *unc-103(0); egl-2(0)* and *slo-1(rg432)* animals (Figure 4, C and D). This indicates that the lack of EGL-2 and UNC-103 activity has a synthetic interaction with the *rg432* allele to globally reduce *slo-1* mRNA abundance even more so than the *rg432* allele alone. However, the behavior of *unc-103(0); egl-2(0) slo-1(rg432)* triple mutants was not worse than *unc-103(0); egl-2(0)* double mutants, but rather, resembles *unc-103(0)* single mutants. This suggests that the amount of *slo-1* mRNA cannot be solely rate limiting in controlling behavior. Thus a complex interplay between the consequences of the three mutant alleles might have a pleiotropic effect on other genes that control general neural and muscle excitability.

Finally, we asked whether a molecule that is known to pleiotropically affect neural and muscle cell excitability also influences *slo-1*, *egl-2*, and *unc-103* mRNA abundance. CaMKII is a calcium-activated protein and is known to influence gene expression (Kapiloff *et al.* 1991; Ramirez *et al.* 1997; Hughes *et al.* 2001; Zhang *et al.* 2004; Ronkainen *et al.* 2011). In *C. elegans*, loss-of-function mutations in CaMKII/*unc-43* have profound deleterious effects on virtually every behavior of the worm (Reiner *et al.* 1999). The *unc-43(sy574)* allele we previously isolated causes males to display the Prc phenotype, but does not have any gross effect on other behaviors in males or hermaphrodites (LeBoeuf *et al.* 2007, 2011). We therefore asked whether the *sy574* allele, which outwardly appears to affect one behavior, influences global *slo-1*, *egl-2*, and *unc-103* mRNA abundance. We discovered that the transcript amount of all three K⁺ channels was decreased in animals that contain the *unc-43(sy574)* allele (Figure 4). This result indicates that despite the phenotypic mildness of the *unc-43(sy574)* allele, it has broad effects of mRNA levels, and that *slo-1*, *egl-2*, and *unc-103* expression is directly or indirectly connected with CaMKII function.

***slo-1(rg432)* preferentially affects genes involved in regulating cell excitability in males**

The above experiments do not explain how the *slo-1(rg432)* allele, which results in decreased *slo-1* transcript levels in hermaphrodite and male populations and decreased *egl-2*

and *unc-103* mRNA levels in mixed-staged populations, is able to reduce spicule protraction in *unc-103(0)*; *egl-2(0)* males. Decreasing the pool of available transcripts should have a negative impact on the amount of protein produced, and consequently a cell's ability to maintain its polarized state. One possibility is that, as a response to decreased *slo-1* transcript levels, cells generally lower the amount of other components that promote cell depolarization, thus resulting in a more stable system. To test this hypothesis, we measured the transcript levels of genes involved in regulating the excitability of cells used in male mating behavior and also of genes involved in more general cell processes. We analyzed *acr-18* [nicotinic acetylcholine receptor (nAChR) α -subunit], *gar-3* (ACh receptor coupled to $g\alpha_q$), *unc-29* (nAChR non- α -subunit), *unc-43* (CaMKII), and *cat-2* (tyrosine hydroxylase) as candidate genes that are enriched in excitable cells (Sulston *et al.* 1975; Fleming *et al.* 1997; Hwang *et al.* 1999; Lints and Emmons 1999; Reiner *et al.* 1999; Sze *et al.* 2000; Garcia *et al.* 2001; Mongan *et al.* 2002; LeBoeuf *et al.* 2007; Liu *et al.* 2007b, 2011). Neither *acr-18* nor *cat-2* transcript levels were increased in males as compared to hermaphrodites (Figure 3, C and D), although both were reduced in *slo-1(rg432)* males. While *C. elegans* males have many more excitable cells than hermaphrodites, only a few of these express *cat-2*, which could account for the lack of difference between sexes. *acr-18* is expressed in many male-specific neurons and muscles (Liu *et al.* 2011); however, the *acr-18* mRNA was slightly, but not significantly increased in males as compared to hermaphrodites. The *acr-18* levels could be reduced in non-sex-specific cells in males, resulting in transcript levels that are not significantly different from hermaphrodites.

In contrast to *acr-18* and *cat-2* and similar to *slo-1*, the genes *gar-3*, *unc-29*, and *unc-43* showed greatly increased transcript levels in the male; this increase was abolished in *slo-1(rg432)* worms (Figure 3, C and D). Thus, in response to the *rg432* allele, cells appear to downregulate other molecules, along with the SLO-1 channel, involved in regulating cell excitability.

In addition to genes enriched in excitable cells, we also looked at the transcript levels of genes with more general expression patterns: *let-363* (Tor-like kinase), *sir-2.1* (histone deacetylase), *cdc-42* (RHO GTPase), and *pmp-3* [ATP-binding cassette (ABC) transporter] (Chen *et al.* 1993; McKim *et al.* 1993; Frye 2000; Hoogewijs *et al.* 2008). Relative to hermaphrodites, male transcript levels were increased for *let-363* and *sir-2.1* (Figure 3, C and D). mRNA levels for *let-363*, *sir-2.1*, and *pmp-3*, but not *cdc-42*, dropped in *slo-1(rg432)* males; however, the differences were not as pronounced as compared to transcripts enriched in excitable cells (Figure 3, C and D). In addition, transcript levels for *let-363*, *sir-2.1*, and *pmp-3* were increased in *slo-1(rg432)* hermaphrodites, in contrast to the lower transcript levels of the analyzed excitable cell-enriched genes. Taken together, these data suggest that the *slo-1(rg432)* allele broadly affects the levels of mRNA encoded by genes that

regulate cell excitability more so than genes with more general cell functions.

Discussion

The excitability of neurons and muscles need to be fine-tuned for the optimal performance of complex behaviors. *C. elegans* male mating is a demanding behavior requiring coordinated feedback mechanisms to promote the multiple steps of mating. Failure of any one step leads to unsuccessful mating. For example, full spicule protraction occurs when the spicules penetrate the vulval slit and is inhibited until the correct cues are received from all inputs. A breakdown in regulation leads to promiscuous protraction at ectopic areas, thus reducing successful sperm transfer. Spicule protraction depends on the integration of signaling from neurons and muscles, including the SPC motor neuron and the dorsal and ventral protractor muscles. The SPC releases acetylcholine (ACh) onto the protractors, initiating contraction. Gap junctions between the protractors and other muscles and neurons in the circuit help reduce the excitability threshold of the protractors, so they can respond to the ACh released by the SPC neurons (Garcia *et al.* 2001; Liu *et al.* 2011). BK/SLO-1, ERG-like/UNC-103, and EAG/EGL-2 K^+ channels attenuate circuit activity until sufficient cues have been received (Figure 5A).

An important contributor to regulating male mating circuit excitability is BK channel/*slo-1*. *slo-1* mutants display ectopic spicule protraction, which can be rescued with specific *slo-1* isoforms. Full rescue is achieved by shortening the distance between the two regulator of K^+ channel conductance (RCK) sites. RCK sites are proposed to mediate calcium's gating of the channel (Lee and Cui 2010; Yuan *et al.* 2010), a modification that is common across species (Fodor and Aldrich 2009). Similarly, isoform-specific rescue of flight was shown in *Drosophila* (Brenner *et al.* 2000). Across different species, age, cell type, and stress impact which isoforms of *slo-1* are expressed (Tseng-Crank *et al.* 1994; Xie and McCobb 1998; MacDonald *et al.* 2006; Kim *et al.* 2010). In *C. elegans* SLO-1, inclusion of the exon at splice site C1 results in channels with slower activation when expressed in *Xenopus oocytes* (Johnson *et al.* 2011). Our data suggest a functional consequence for modifying this linker region. The subtle changes in *slo-1* that promote the channel's ability to inhibit precocious spicule protraction highlight the large impact that small changes in channel structure have on behavior.

In addition to identifying an isoform change that promotes reduction of sex muscle excitability, we identified an intronic point mutation [*slo-1(rg432)*] that significantly reduces *slo-1* transcript levels and sex muscle excitability. *slo-1(rg432)* also reduces the transcript levels of genes involved in regulating cell excitability. This suggests that the compensatory response to reduced *slo-1* levels is to diminish the levels of other genes involved in cell depolarization. Many human diseases are the result of intronic point mutations (Watanabe *et al.* 2000; Lamba *et al.* 2002; Keren *et al.*

Figure 5 K⁺ channel regulation of sex muscle excitability. (A) Abbreviated cartoon of the male tail. Sex muscles are indicated in gray. The ERG, EAG, and BK K⁺ channels inhibit spicule protraction until the male has penetrated the hermaphrodite vulva. (B) Diagram of part of the male mating circuit. The SPC motor neuron innervates the protractor muscles and releases constant low levels of acetylcholine (ACh) to help maintain sex muscle excitability. ACh activates muscarinic ACh receptors, releasing G α_q that results in intracellular increase in Ca²⁺ (Liu *et al.* 2007b). This in turn activates CaMKII, which increases the transcription of K⁺ channel genes to help lower the sex muscle excitability.

2010); identifying ectopic genetic responses provides potential therapeutic routes to alleviate the effects of such diseases.

SLO-1 does not regulate male mating circuit excitability alone but functions with the EAG family K⁺ channels EAG/EGL-2 and ERG/UNC-103. By examining how these K⁺ channels impact one another, we elucidated their coordinated effort to regulate cell excitability and behavior. Approximately 70–80% of males lacking *slo-1* display premature spicule protraction; this fraction does not change when both *egl-2* and *unc-103* are removed. However, removing only one results in males with reduced spontaneous muscle spasms. Thus, *egl-2* or *unc-103* can partially compensate for *slo-1* deficiency. These three channels have also been shown to mediate synapse formation in *Drosophila* (Budnik

et al. 1990; Berke *et al.* 2006; Lee and Wu 2010), highlighting the conserved mechanisms used to set up cell excitability and produce behavior.

Studying transcript levels in different K⁺ channel mutants indicates *egl-2* stabilizes *unc-103* and *slo-1* mRNA. In *egl-2(0)* males, *unc-103* and *slo-1* mRNA levels are lower than wild type. Genetic interactions indicate *egl-2* allows *slo-1* to compensate for the loss of *unc-103* function. *unc-103(0)*; *egl-2(0)* males display a higher instance of abnormal spicule protraction than do *unc-103(0)* males. The difference is likely due to an upregulation of *slo-1* in an *unc-103(0)* background. *slo-1* mRNA stability increases in an *unc-103(0)* background, as does expression of a reporter gene from the *slo-1* promoter. If the increase in transcription promotes protein synthesis, more EGL-2 and SLO-1 in the membrane could be compensating for UNC-103 deficiency. Additionally, the effect of the *unc-103(sy557)* neomorphic allele on *egl-2* and *slo-1* function provides further evidence of an interaction between the three K⁺ channels. *unc-103(sy557)* induces spicule protraction at a level similar to *unc-103(0)*; *egl-2(0) slo-1(lf)* and the increased levels of *slo-1* and *egl-2* transcription seen in *unc-103(0)* males are reversed in *unc-103(sy557)* mutants. These data suggest that *unc-103(sy557)* negatively impacts *egl-2* and *slo-1*, and conversely, these three K⁺ channels function interdependently in wild-type circuits. Finally, *egl-2* needs to be absent for *unc-103* to compensate for *slo-1* deficiency: the instance of spontaneous spicule protraction drops 40% from *slo-1(lf)* males compared to *egl-2(0) slo-1(lf)* males. These results suggest SLO-1 activity is dependent upon EGL-2, while EGL-2 attenuates UNC-103 compensatory function.

In *C. elegans*, EAG and BK channels also interact to down-regulate sex muscle excitability when the young adult males undergo a period of starvation, presumably to direct the males' behavior from copulation and toward food acquisition (Gruninger *et al.* 2006; LeBoeuf *et al.* 2011). We previously reported that EAG K⁺ channels play a prominent role during food deprivation (LeBoeuf *et al.* 2007, 2011); here, we show that BK channels are also important. When *slo-1(lf)* males are food deprived, spontaneous spicule protraction is partially suppressed in an EAG K⁺ channel-dependent manner. Modifying EAG K⁺ channel function through a neomorphic genetic mutation enhances the effect of food deprivation to suppress *slo-1(lf)*-induced muscle spasms.

Modifying K⁺ channel function is one way for the circuit to regulate excitability; adjusting calcium levels is another. Calcium is a main transducer of excitatory signals and a circuit must maintain tight control of intracellular calcium levels. The Ca²⁺-sensitive BK channels provide one method of regulatory response; Ca²⁺-activated kinases are another. A candidate is CaMKII, which acts as a repressor of excitability in the male mating circuit (LeBoeuf *et al.* 2007). K⁺ channels in the circuit set up the delicate balance necessary for reproduction, and CaMKII may influence their transcription (Figure 5B). CaMKII's role in regulating transcription is well

documented (Li *et al.* 2010; Ely *et al.* 2011; Oruganti *et al.* 2011), especially in cardiac muscle (Zhang *et al.* 2004; Backs *et al.* 2006; Little *et al.* 2007). In *C. elegans*, it is possible CaMKII is regulating the transcription of *slo-1*, *egl-2*, and *unc-103*, since all three transcript levels are decreased in a CaMKII/*unc-43(lf)* mutant. CaMKII also acts through EAG K⁺ channels during starvation and both EAG and ERG K⁺ channels under standard conditions (LeBoeuf *et al.* 2007, 2011). However, there is genetic evidence to suggest that CaMKII acts through additional K⁺ channels in the male. Nearly 100% of *unc-103(0)*; *unc-43(lf)* (LeBoeuf *et al.* 2007) and *unc-43(lf)*; *slo-1(lf)* double mutant males (this article) spontaneously protract their spicules. However, only 79% of triple K⁺ channel mutant males display abnormal behavior. This indicates that there are additional unidentified molecules through which CaMKII is acting to regulate male sex muscle excitability.

Acknowledgments

We thank LaShundra Rodgers for technical assistance and Benjamin Russo, Daisy Gualberto, Liusuo Zhang, and James Midkiff for critical reading of the manuscript. *C. elegans* strains were provided by the *Caenorhabditis* Genetic Center, which is funded by the National Center for Research Resources, National Institutes of Health. This work was supported by the Howard Hughes Medical Institute.

Literature Cited

- Anderson, P., 1995 Mutagenesis, pp. 31–58 in *Methods in Cell Biology, Caenorhabditis elegans: Modern Biological Analysis of an Organism*, edited by H. F. Epstein and D. C. Shakes. Academic Press, San Diego.
- Backs, J., K. Song, S. Bezprozvannaya, S. Chang, and E. N. Olson, 2006 CaM kinase II selectively signals to histone deacetylase 4 during cardiomyocyte hypertrophy. *J. Clin. Invest.* 116: 1853–1864.
- Bargmann, C. I., 1998 Neurobiology of the *Caenorhabditis elegans* genome. *Science* 282: 2028–2033.
- Berke, B. A., J. Lee, I. F. Peng, and C. F. Wu, 2006 Sub-cellular Ca²⁺ dynamics affected by voltage- and Ca²⁺-gated K⁺ channels: regulation of the soma-growth cone disparity and the quiescent state in *Drosophila* neurons. *Neuroscience* 142: 629–644.
- Brenner, R., J. Y. Yu, K. Srinivasan, L. Brewer, J. L. Larimer *et al.*, 2000 Complementation of physiological and behavioral defects by a slowpoke Ca(2+) -activated K(+) channel transgene. *J. Neurochem.* 75: 1310–1319.
- Brenner, S., 1974 The genetics of *Caenorhabditis elegans*. *Genetics* 77: 71–94.
- Budnik, V., Y. Zhong, and C. F. Wu, 1990 Morphological plasticity of motor axons in *Drosophila* mutants with altered excitability. *J. Neurosci.* 10: 3754–3768.
- Carre-Pierrat, M., K. Grisoni, K. Gieseler, M. C. Mariol, E. Martin *et al.*, 2006 The SLO-1 BK channel of *Caenorhabditis elegans* is critical for muscle function and is involved in dystrophin-dependent muscle dystrophy. *J. Mol. Biol.* 358: 387–395.
- Chen, W., H. H. Lim, and L. Lim, 1993 The CDC42 homologue from *Caenorhabditis elegans*. Complementation of yeast mutation. *J. Biol. Chem.* 268: 13280–13285.
- Davies, A. G., J. T. Pierce-Shimomura, H. Kim, M. K. VanHoven, T. R. Thiele *et al.*, 2003 A central role of the BK potassium channel in behavioral responses to ethanol in *C. elegans*. *Cell* 115: 655–666.
- Du, W., J. F. Bautista, H. Yang, A. Diez-Sampedro, S. A. You *et al.*, 2005 Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder. *Nat. Genet.* 37: 733–738.
- Ely, H. A., P. L. Mellon, and D. Coss, 2011 GnRH induces the c-Fos gene via phosphorylation of SRF by the calcium/calmodulin kinase II pathway. *Mol. Endocrinol.* 25: 669–680.
- Fleming, J. T., M. D. Squire, T. M. Barnes, C. Tornoe, K. Matsuda *et al.*, 1997 *Caenorhabditis elegans* levamisole resistance genes *lev-1*, *unc-29*, and *unc-38* encode functional nicotinic acetylcholine receptor subunits. *J. Neurosci.* 17: 5843–5857.
- Fodor, A. A., and R. W. Aldrich, 2009 Convergent evolution of alternative splices at domain boundaries of the BK channel. *Annu. Rev. Physiol.* 71: 19–36.
- Frye, R. A., 2000 Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochem. Biophys. Res. Commun.* 273: 793–798.
- Garcia, L. R., and P. W. Sternberg, 2003 *Caenorhabditis elegans* UNC-103 ERG-like potassium channel regulates contractile behaviors of sex muscles in males before and during mating. *J. Neurosci.* 23: 2696–2705.
- Garcia, L. R., P. Mehta, and P. W. Sternberg, 2001 Regulation of distinct muscle behaviors controls the *C. elegans* male's copulatory spicules during mating. *Cell* 107: 777–788.
- Glauser, D. A., B. E. Johnson, R. W. Aldrich, and M. B. Goodman, 2011 Intragenic alternative splicing coordination is essential for *Caenorhabditis elegans slo-1* gene function. *Proc. Natl. Acad. Sci. USA* 108: 20790–20795.
- Granato, M., H. Schnabel, and R. Schnabel, 1994 *pha-1*, a selectable marker for gene transfer in *C. elegans*. *Nucleic Acids Res.* 22: 1762–1763.
- Grimm, P. R., and S. C. Sansom, 2010 BK channels and a new form of hypertension. *Kidney Int.* 78: 956–962.
- Gruninger, T. R., D. G. Gualberto, B. LeBoeuf, and L. R. Garcia, 2006 Integration of male mating and feeding behaviors in *Caenorhabditis elegans*. *J. Neurosci.* 26: 169–179.
- Hawasli, A. H., O. Saifee, C. Liu, M. L. Nonet, and C. M. Crowder, 2004 Resistance to volatile anesthetics by mutations enhancing excitatory neurotransmitter release in *Caenorhabditis elegans*. *Genetics* 168: 831–843.
- Hodgkin, J. A., H. R. Horvitz, and S. Brenner, 1979 Non-disjunction mutants of the nematode *Caenorhabditis elegans*. *Genetics* 91: 67–94.
- Hodgkin, J., A. Papp, R. Pulak, V. Ambros, and P. Anderson, 1989 A new kind of informational suppression in the nematode *Caenorhabditis elegans*. *Genetics* 123: 301–313.
- Hoogewijs, D., K. Houthoofd, F. Matthijssens, J. Vandesompele, and J. Vanfleteren, 2008 Selection and validation of a set of reliable reference genes for quantitative *sod* gene expression analysis in *C. elegans*. *BMC Mol. Biol.* 9: 9.
- Hughes, K., S. Edin, A. Antonsson, and T. Grundstrom, 2001 Calmodulin-dependent kinase II mediates T cell receptor/CD3- and phorbol ester-induced activation of I κ B kinase. *J. Biol. Chem.* 276: 36008–36013.
- Hwang, J. M., D. J. Chang, U. S. Kim, Y. S. Lee, Y. S. Park *et al.*, 1999 Cloning and functional characterization of a *Caenorhabditis elegans* muscarinic acetylcholine receptor. *Receptors Channels* 6: 415–424.
- Johnson, B. E., D. A. Glauser, E. S. Dan-Glauser, D. B. Halling, R. W. Aldrich *et al.*, 2011 Alternatively spliced domains interact to regulate BK potassium channel gating. *Proc. Natl. Acad. Sci. USA* 108: 20784–20789.

- Kapiloff, M. S., J. M. Mathis, C. A. Nelson, C. R. Lin, and M. G. Rosenfeld, 1991 Calcium/calmodulin-dependent protein kinase mediates a pathway for transcriptional regulation. *Proc. Natl. Acad. Sci. USA* 88: 3710–3714.
- Keren, H., M. Donyo, D. Zeevi, C. Maayan, T. Pupko *et al.*, 2010 Phosphatidylserine increases IKBKAP levels in familial dysautonomia cells. *PLoS ONE* 5: e15884.
- Kim, H., J. T. Pierce-Shimomura, H. J. Oh, B. E. Johnson, M. B. Goodman *et al.*, 2009 The dystrophin complex controls BK channel localization and muscle activity in *Caenorhabditis elegans*. *PLoS Genet.* 5: e1000780.
- Kim, J. M., R. Beyer, M. Morales, S. Chen, L. Q. Liu *et al.*, 2010 Expression of BK-type calcium-activated potassium channel splice variants during chick cochlear development. *J. Comp. Neurol.* 518: 2554–2569.
- Lamba, J. K., Y. S. Lin, E. G. Schuetz, and K. E. Thummel, 2002 Genetic contribution to variable human CYP3A-mediated metabolism. *Adv. Drug Deliv. Rev.* 54: 1271–1294.
- LeBoeuf, B., T. R. Gruninger, and L. R. Garcia, 2007 Food deprivation attenuates seizures through CaMKII and EAG K⁺ Channels. *PLoS Genet.* 3: e156.
- LeBoeuf, B., X. Guo, and L. R. Garcia, 2011 The effects of transient starvation persist through direct interactions between CaMKII and ether-a-go-go K⁺ channels in *C. elegans* males. *Neuroscience* 175: 1–17.
- Lee, J., and C. F. Wu, 2010 Orchestration of stepwise synaptic growth by K⁺ and Ca²⁺ channels in *Drosophila*. *J. Neurosci.* 30: 15821–15833.
- Lee, U. S., and J. Cui, 2010 BK channel activation: structural and functional insights. *Trends Neurosci.* 33: 415–423.
- Li, H., W. Li, A. K. Gupta, P. J. Mohler, M. E. Anderson *et al.*, 2010 Calmodulin kinase II is required for angiotensin II-mediated vascular smooth muscle hypertrophy. *Am. J. Physiol. Heart Circ. Physiol.* 298: H688–H698.
- Lints, R., and S. W. Emmons, 1999 Patterning of dopaminergic neurotransmitter identity among *Caenorhabditis elegans* ray sensory neurons by a TGFbeta family signaling pathway and a Hox gene. *Development* 126: 5819–5831.
- Little, G. H., Y. Bai, T. Williams, and C. Poizat, 2007 Nuclear calcium/calmodulin-dependent protein kinase IIdelta preferentially transmits signals to histone deacetylase 4 in cardiac cells. *J. Biol. Chem.* 282: 7219–7231.
- Liu, J., M. Asuncion-Chin, P. Liu, and A. M. Dopico, 2006 CaM kinase II phosphorylation of slo Thr107 regulates activity and ethanol responses of BK channels. *Nat. Neurosci.* 9: 41–49.
- Liu, K. S., and P. W. Sternberg, 1995 Sensory regulation of male mating behavior in *Caenorhabditis elegans*. *Neuron* 14: 79–89.
- Liu, Q., B. Chen, Q. Ge, and Z. W. Wang, 2007a Presynaptic Ca²⁺/calmodulin-dependent protein kinase II modulates neurotransmitter release by activating BK channels at *Caenorhabditis elegans* neuromuscular junction. *J. Neurosci.* 27: 10404–10413.
- Liu, Y., B. LeBoeuf, and L. R. Garcia, 2007b G alpha(q)-coupled muscarinic acetylcholine receptors enhance nicotinic acetylcholine receptor signaling in *Caenorhabditis elegans* mating behavior. *J. Neurosci.* 27: 1411–1421.
- Liu, Y., B. LeBoeuf, X. Guo, P. A. Correa, D. G. Gualberto *et al.*, 2011 A cholinergic-regulated circuit coordinates the maintenance and bi-stable states of a sensory-motor behavior during *Caenorhabditis elegans* male copulation. *PLoS Genet.* 7: e1001326.
- Livak, K. J., and T. D. Schmittgen, 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)). *Method. Methods* 25: 402–408.
- Longman, D., R. H. Plasterk, I. L. Johnstone, and J. F. Caceres, 2007 Mechanistic insights and identification of two novel factors in the *C. elegans* NMD pathway. *Genes Dev.* 21: 1075–1085.
- MacDonald, S. H., P. Ruth, H. G. Knaus, and M. J. Shipston, 2006 Increased large conductance calcium-activated potassium (BK) channel expression accompanied by STREX variant downregulation in the developing mouse CNS. *BMC Dev. Biol.* 6: 37.
- Maquat, L. E., 2004 Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics. *Nat. Rev. Mol. Cell Biol.* 5: 89–99.
- McKim, K. S., K. Peters, and A. M. Rose, 1993 Two types of sites required for meiotic chromosome pairing in *Caenorhabditis elegans*. *Genetics* 134: 749–768.
- Mello, C. C., J. M. Kramer, D. Stinchcomb, and V. Ambros, 1991 Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* 10: 3959–3970.
- Mongan, N. P., A. K. Jones, G. R. Smith, M. S. Sansom, and D. B. Sattelle, 2002 Novel alpha7-like nicotinic acetylcholine receptor subunits in the nematode *Caenorhabditis elegans*. *Protein Sci.* 11: 1162–1171.
- Oruganti, S. R., S. Edin, C. Grundstrom, and T. Grundstrom, 2011 CaMKII targets Bcl10 in T-cell receptor induced activation of NF-κB. *Mol. Immunol.* 48: 1448–1460.
- Park, E. C., and H. R. Horvitz, 1986 Mutations with dominant effects on the behavior and morphology of the nematode *Caenorhabditis elegans*. *Genetics* 113: 821–852.
- Ramirez, M. T., X. L. Zhao, H. Schulman, and J. H. Brown, 1997 The nuclear deltaB isoform of Ca²⁺/calmodulin-dependent protein kinase II regulates atrial natriuretic factor gene expression in ventricular myocytes. *J. Biol. Chem.* 272: 31203–31208.
- Reiner, D. J., D. Weinshenker, and J. H. Thomas, 1995 Analysis of dominant mutations affecting muscle excitation in *Caenorhabditis elegans*. *Genetics* 141: 961–976.
- Reiner, D. J., E. M. Newton, H. Tian, and J. H. Thomas, 1999 Diverse behavioural defects caused by mutations in *Caenorhabditis elegans unc-43* CaM kinase II. *Nature* 402: 199–203.
- Reiner, D. J., D. Weinshenker, H. Tian, J. H. Thomas, K. Nishiwaki *et al.*, 2006 Behavioral genetics of *Caenorhabditis elegans unc-103*-encoded erg-like K(+) channel. *J. Neurogenet.* 20: 41–66.
- Ronkainen, J. J., S. L. Hanninen, T. Korhonen, J. T. Koivumaki, R. Skoumal *et al.*, 2011 Ca²⁺-calmodulin-dependent protein kinase II represses cardiac transcription of the L-type calcium channel 1C-subunit gene (Cacna1c) by DREAM translocation. *J. Physiol.* 589: 2669–2686.
- Salkoff, L., A. Butler, G. Ferreira, C. Santi, and A. Wei, 2006 High-conductance potassium channels of the SLO family. *Nat. Rev. Neurosci.* 7: 921–931.
- Sanguinetti, M. C., C. Jiang, M. E. Curran, and M. T. Keating, 1995 A mechanistic link between an inherited and an acquired cardiac arrhythmia: HERG encodes the IKr potassium channel. *Cell* 81: 299–307.
- Santi, C. M., A. Yuan, G. Fawcett, Z. W. Wang, A. Butler *et al.*, 2003 Dissection of K⁺ currents in *Caenorhabditis elegans* muscle cells by genetics and RNA interference. *Proc. Natl. Acad. Sci. USA* 100: 14391–14396.
- Schmitt, N., M. Schwarz, A. Peretz, I. Abitbol, B. Attali *et al.*, 2000 A recessive C-terminal Jervell and Lange-Nielsen mutation of the KCNQ1 channel impairs subunit assembly. *EMBO J.* 19: 332–340.
- Schnabel, H., and R. Schnabel, 1990 An organ-specific differentiation gene, *pha-1*, from *Caenorhabditis elegans*. *Science* 250: 686–688.
- Sulston, J., M. Dew, and S. Brenner, 1975 Dopaminergic neurons in the nematode *Caenorhabditis elegans*. *J. Comp. Neurol.* 163: 215–226.

- Sulston, J. E., D. G. Albertson, and J. N. Thomas, 1980 The *Caenorhabditis elegans* male: postembryonic development of non-gonadal structures. *Dev. Biol.* 78: 542–576.
- Sulston, J. E., E. Schierenberg, J. G. White, and J. N. Thomson, 1983 The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* 100: 64–119.
- Sze, J. Y., M. Victor, C. Loer, Y. Shi, and G. Ruvkun, 2000 Food and metabolic signalling defects in a *Caenorhabditis elegans* serotonin-synthesis mutant. *Nature* 403: 560–564.
- Terskikh, A., A. Fradkov, G. Ermakova, A. Zaisky, P. Tan *et al.*, 2000 “Fluorescent timer”: protein that changes color with time. *Science* 290: 1585–1588.
- Torres, Y. P., F. J. Morera, I. Carvacho, and R. Latorre, 2007 A marriage of convenience: beta-subunits and voltage-dependent K⁺ channels. *J. Biol. Chem.* 282: 24485–24489.
- Tseng-Crank, J., C. D. Foster, J. D. Krause, R. Mertz, N. Godinot *et al.*, 1994 Cloning, expression, and distribution of functionally distinct Ca(2+)-activated K⁺ channel isoforms from human brain. *Neuron* 13: 1315–1330.
- Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy *et al.*, 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3: RESEARCH0034.
- Wang, Z. W., O. Saifee, M. L. Nonet, and L. Salkoff, 2001 SLO-1 potassium channels control quantal content of neurotransmitter release at the *C. elegans* neuromuscular junction. *Neuron* 32: 867–881.
- Watanabe, Y., S. Kato, Y. Adachi, and K. Nakashima, 2000 Frame-shift, nonsense and non amino acid altering mutations in SOD1 in familial ALS: report of a Japanese pedigree and literature review. *Amyotroph. Lateral Scler. Other Motor Neuron Disord.* 1: 251–258.
- Weinshenker, D., G. Garriga, and J. H. Thomas, 1995 Genetic and pharmacological analysis of neurotransmitters controlling egg laying in *C. elegans*. *J. Neurosci.* 15: 6975–6985.
- Weinshenker, D., A. Wei, L. Salkoff, and J. H. Thomas, 1999 Block of an *ether-a-go-go*-like K(+) channel by imipramine rescues *egl-2* excitation defects in *Caenorhabditis elegans*. *J. Neurosci.* 19: 9831–9840.
- Werner, M. E., P. Zvara, A. L. Meredith, R. W. Aldrich, and M. T. Nelson, 2005 Erectile dysfunction in mice lacking the large-conductance calcium-activated potassium (BK) channel. *J. Physiol.* 567: 545–556.
- Xie, J., and D. P. McCobb, 1998 Control of alternative splicing of potassium channels by stress hormones. *Science* 280: 443–446.
- Yuan, P., M. D. Leonetti, A. R. Pico, Y. Hsiung, and R. MacKinnon, 2010 Structure of the human BK channel Ca²⁺-activation apparatus at 3.0 Å resolution. *Science* 329: 182–186.
- Zhang, T., S. Miyamoto, and J. H. Brown, 2004 Cardiomyocyte calcium and calcium/calmodulin-dependent protein kinase II: friends or foes? *Recent Prog. Horm. Res.* 59: 141–168.

Communicating editor: R. Anholt