Caenorhabditis elegans UNC-103 ERG-Like Potassium Channel Regulates Contractile Behaviors of Sex Muscles in Males before and during Mating

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During mating behavior the *Caenorhabditis elegans* male must regulate periodic and prolonged protractor muscle contractions to insert his copulatory spicules into his mate. The protractors undergo periodic contractions to allow the spicules to reattempt insertion if a previous thrust failed to breach the vulva. When the spicule tips penetrate the vulva, the protractors undergo prolonged contraction to keep the spicules inside the hermaphrodite until sperm transfer is complete. To understand how these contractions are regulated, we isolated EMS-induced mutations that cause males to execute prolonged contraction inappropriately. Loss-of-function mutations in the *unc-103* ERG-like K$^+$ channel gene cause the protractor muscles to contract in the absence of mating stimulation. *unc-103*-induced spicule protraction can be suppressed by killing the SPC motor neurons and the anal depressor muscle: cells that directly contact the protractors. Also, reduction in acetylcholine suppresses *unc-103*-induced protraction, suggesting that UNC-103 keeps cholinergic neurons from stimulating the protractors before mating behavior. UNC-103 also regulates the timing of spicule protraction during mating behavior. *unc-103* males that do not display mating-independent spicule protraction show abnormal spicule insertion behavior during sex. In contrast to wild-type males, *unc-103* mutants execute prolonged contractions spontaneously within sequences of periodic protractor contractions. The premature prolonged contractions cause the spicules to extend from the male tail before the spicule tips penetrate the vulva. These observations demonstrate that *unc-103* controls various aspects of spicule function.

Key words: Caenorhabditis elegans; mating behavior; *unc-103*; ERG; K$^+$ channel; L-type voltage-gated calcium channels

Introduction

Analysis of *Drosophila* behavioral mutants led to the first *in vivo* correlation among K$^+$ channel genes such as *shaker* (Kamb et al., 1987; Temple et al., 1987; Pongs et al., 1988), *slowpoke* (Atkinson et al., 1991), *ether-a-go-go* (Kaplan and Trout, 1969; Warmke et al., 1991), and *seizure/erg* (Titus et al., 1997; Wang et al., 1997) and their roles in specific motor outputs (Papazian et al., 1988). Mutations that affect these K$^+$ channels also perturb more complex behaviors such as habituation of escape response (Engel and Wu, 1998), courtship behavior (Griffith et al., 1994), and olfaction (Cowan and Siegel, 1986).

In *Caenorhabditis elegans* genetic screens also have identified K$^+$ channel genes that regulate different behaviors. Gain-of-function mutations in the K$^+$ channel genes like *unc-103* (the homolog of *seizure/erg*) (Reiner et al., 1999; J. H. Thomas, personal communication), *egl-36* (the homolog of *shaw*) (Elkes et al., 1997; Johnstone et al., 1997), *egl-2* (the homolog of *ether-a-go-go*) (Weinshenker et al., 1999), *twk-18* (two-P domain K$^+$ channel) (Kunkel et al., 2000), and *exp-2* (a novel K$_v$ channel) (Davis et al., 1999) reduce locomotion, defecation, and egg-laying behaviors (Trent et al., 1983; Park and Horvitz, 1986; Thomas, 1990; Reiner et al., 1995). Loss-of-function mutations in *slo-1* (calcium-activated, voltage-gated K$^+$ channel) suppress the general lethargy of *unc-64* (syntaxin) mutants (Wang et al., 2001).

C. elegans male mating behavior is a complex behavior that requires the coordination of different motor outputs (Ward and Carrel, 1979; Loer and Kenyon, 1993; Barker, 1994; Liu and Sternberg, 1995; Barr and Sternberg, 1999; Garcia et al., 2001; Simon and Sternberg, 2002). To sire progeny, the male must insert his copulatory spicules into his mate; this process requires the regulation of two types of spicule protractor muscle contractions. During spicule insertion behavior the PCB and PCC postcloacal sensory neurons signal the protractor muscles to contract and relax at a frequency of 7–9 Hz, causing the spicules to prod the vulval slit repeatedly. When the spicules penetrate the vulva, the SPC motor neurons signal periodic contractions to cease and cause the protractor muscles to remain contracted until sperm transfer is completed (Garcia et al., 2001).

To understand how distinct motor outputs are regulated, we isolated mutations that induce spontaneous spicule protraction. We find that mutations in *unc-103*, the worm homolog of *Drosophila seizure/erg*-encoded and the human *h-erg*-encoded delayed inward rectifying voltage-gated K$^+$ channel, affect the spicule insertion step (Sanguinetti et al., 1995; Trudeau et al., 1995; Titus et al., 1997; Wang et al., 1997; Reiner et al., 1999; J. H. Thomas, personal communication).

h-erg encodes the cardiac I_{Kr} K$^+$ channel that facilitates cardiac rhythms (Sanguinetti et al., 1995). HERG channels display fast inactivation that reduce potassium efflux during depolarization and display rapid recovery during repolarization (Trudeau et al., 1995). In humans and flies, mutations in *erg* have profound effects on the organism. Individuals that contain *h-erg* mutations can suffer from lethal heart arrhythmias (Curran et al., 1995), and flies with *sei/erg* mutations display temperature-induced seizures (Titus et al., 1997; Wang et al., 1997). In contrast, we find that in *C. elegans* males UNC-103 has a specific role in mating behavior, and it regulates sex muscle contractions before and during copulation.

Materials and Methods

Strains. All strains used in this study contain *him-5(e1490)* on LGV (Hodgkin et al., 1979). We maintained the strains by using standard culture conditions (Brenner, 1974). Our wild-type reference strain, PS1395, contains *plg-1(e2001)* on LGIII. This allele allows males to secrete a gelatinous plug over the vulva after sperm transfer (Hodgkin and Doniach, 1997). We also used the following alleles in this study: *unc-38(sy576)* (Garcia et al., 2001) and *unc-13(e51)* (Brenner, 1974) on LGI; *daf-2(e1370)* (Riddle, 1977), *unc-93(e1500)* (Greenwald and Horvitz, 1980), *unc-79(e1068)* (Hodgkin, 1983), *unc-103(e1597gf)* (Hodgkin, 1983), *unc-103(n1213)* (Park and Horvitz, 1986), *pal-1(e2091)* (Waring and Kenyon, 1990), *dpy-17(e164)* (Brenner, 1974), *pha-1(e2123)* (Schnabel and Schnabel, 1990), *unc-49(e382)* (Brenner, 1974), and *unc-64(e246)* (Brenner, 1974) on LGIII; *unc-31(e169)* (Brenner, 1974) and *egl-19(n582)* (Trent et al., 1983) on LGIV; *unc-68(r1158)* (Maryon et al., 1996) on LGV; *cat-1(e1111)* (Sulston et al., 1975) and *unc-2(e55)* (Brenner, 1974) on LGX. For single nucleotide polymorphism (SNP) mapping we used the Hawaiian CB4856 strain as the source of the polymorphisms (Wicks et al., 2001).

Isolation of sy557 *and scoring mating-independent spicule protraction.* To isolate *sy557*, we first mutagenized PS1395 hermaphrodites by using ethyl methanesulfonate (EMS) (Brenner, 1974). After the EMS-mutagenized hermaphrodites produced the F1 generation, we put single F1 hermaphrodites on Petri plates and allowed them to produce the F2 generation. We identified plates in which F2 males and hermaphrodites had general wild-type behavior and in which approximately one-fourth of the males had protruding spicules. We then isolated a homozygous line by picking individual sibling hermaphrodites to separate plates. We kept plates in which the majority of progeny males had protruding spicules. From 10,000 mutagenized gametes we isolated *sy557*, *sy558*, *sy559*, and *sy574*.

To isolate *sy670*, *sy673*, and *sy674*, we followed the procedure of Park and Horvitz (1986). We EMS-mutagenized *unc-103(e1597)*;*him-5(e1490)*; in the F2 generation we isolated hermaphrodites with wild-type behavior. We then scored how many progeny males spontaneously protracted their spicules.

sy557 animals were out-crossed six times to N2 strains. We outcrossed *sy670*, *sy673*, and *sy674* animals to non-mutagenized *unc-103(e1597)* two times before we assayed their behavior. To score mating-independent spicule protraction, we first removed L4 males away from hermaphrodites, putting 20–30 males together on a Petri plate. Then 24 hr later we used a Wild M5A microscope to count how many males had protruding spicules. We continued to score the males for 2 d additionally; however, permanent spicule protraction generally occurred within the first 24 hr of adulthood. Mating-independent spicule protraction is not dependent on population density. The frequency of spicule protraction did not change if individuals were isolated or 150 males were kept together (data not shown). However, if too few males were kept together, the males crawled off the agar and dried on the side of the Petri plate; if too many males were kept together, the males would aggregate, damage the agar surface, and burrow.

Mating efficiency. We adapted the mating efficiency assay from Hodgkin (1983). To measure mating efficiency, we placed six L4 males with six L4 *unc-64(e246)* hermaphrodites on a standard *Escherichia coli* OP50-seeded NG plate. We considered each plate as a single mating trial.

Then 3–4 d later we counted the total number of paralyzed and nonparalyzed cross progeny. Mating efficiencies were calculated as the percentage of cross progeny divided by the number of total progeny.

Mapping of sy557. We used standard mapping procedures to three-factor map *sy557* (Brenner, 1974). In all cases we selected recombinants on the basis of visible hermaphrodite markers and then determined whether recombinant males displayed the *sy557* phenotype. The results from the three-factor mapping experiments are as follows: from *unc-93 dpy-17/sy557* heterozygotes, 9 of 13 Dpy, non-Unc recombinants segregated *sy557*; from *daf-2 unc-79/sy557* heterozygotes, 6 of 6 Daf, non-Unc and 0 of 3 Unc, non-Daf recombinants segregated *sy557*; from *unc-103(e1597) dpy-17/sy557* heterozygotes, 5 of 5 Dpy, non-Unc and 0 of 8 Unc, non-Dpy recombinants segregated *sy557*; from *unc-79 pal-1/sy557* recombinants, 0 of 35 Unc, non-Pal and 1 of 1 Pal, non-Unc recombinants segregated *sy557*. These data suggested that *sy557* mapped near *unc-79* and *unc-103*.

We also used standard SNP mapping procedure to three-factor map *sy557* in relation to SNPs that are located between *unc-93* and *pal-1* (Wicks et al., 2001). The locations of the SNPs in the CB4856 are detailed at http://genome.wustl.edu/projects/celegans. To analyze the SNPs, we used the following primer pairs to amplify the genomic regions of the recombinants: pkP3096 (5′-CCAACTGTCGACGATAGAGATG-3′ and 5′-AAT-TCCGTGTAGCGAATCG-3′); pkP3097 (5′-TTTCAAGACAAGGCAAG-TATCC-3′ and 5′-TTTGATCAATCAGTGGGTCTTG-3′); snp C30D11.4 (5′-TGCTCCACGGATCGACTTGAGAGTGCCGTTGG-3′ and 5′-CGC-TCAACTTCACAATTTCGTAGATGTATATC-3′); snp R10E4.1 (5′-GT-CGGCGGCATCGAGTGAAGCC-3′ and 5′-GTACCATTATTCGAGCT-CAAGGTC-3′); snp C28A5.2 (5′-TTGCATACGTGCCTTTAAGAG-3′ and 5′-CAGAAAGACAGAAAAGGGTCG-3′). We analyzed the SNPs either by DNA sequencing or restriction analysis.

The results from the SNP mapping experiments are as follows: from *daf-2 sy557 dpy-17* (N2)/+ (CB4856) heterozygotes, 1 of 16 *daf-2 sy557+* recombinants contained snp C28A5.2 from CB4856 and 4 of 16 recombinants contained both snp C28A5.2 and snp R10E4.1 from CB4856; from *daf-2 sy557 dpy-17*(N2)/+ (CB4856) heterozygotes, 23 of 24 *daf-2++* recombinants contained the SNPs pkP3096, pkP3097, snp C30D11.4, snp R10E4.1, and snp C28A5.2 from CB4856, and 1 of 24 recombinants contained snp C30D11.4, snp R10E4.1, and snp C28A5.2 from CB4856. These data suggested that *sy557* mapped in a region between snp C30D11.4 and snp R10E4.1

Pharmacology. We dissolved arecoline (purchased from Sigma-Aldrich, St. Louis, MO) in water to make a stock solution of 100 mM. We then serially diluted the stock solution in water as needed. We added 1 ml of the drug to a Pyrex round-bottom, three-well titer dish. Then 5–10 <24 hr adult virgin males were transferred to the drug bath. We observed the males for 5 min with a Wild M5A microscope. The males were considered responsive to the drug if they kept their spicules protracted for ≥10 sec.

Laser ablations. We used standard laser ablation protocols to kill cells in the male tail (Bargmann and Avery, 1995). To ablate neurons, we operated on L4 males that were at a stage in development after the tail ventral hypodermis completely retracted. To ablate the precursor cells P9.p, P10.p, P11.p, F(l/r), and U(l/r), we operated on early L3 males. To rule out any effects that the anesthetic might have on *sy557*-induced behavior, we kept nonoperated cohorts on agar pads soaked with anesthetics similar to the operated males.

unc-103 *reporter construction.* We PCR-amplified an 8.2 kb fragment of DNA that contains 5 kb upstream of the *unc-103* start codon and includes part of the seventh exon of *unc-103*. We used N2 genomic DNA as the template for the PCR and the primers 5′-GGGCACGCCTGCCTAAGG-GATGCCTTAGCTCGGCC-3′ and 5′-CTGGTGAAA TCGGATAAAT-TCTCTG-3′ to amplify the DNA fragment. We then blunt end-ligated the PCR fragment into the *Sma*I site of pPD95.79 (plasmid courtesy of A. Fire, Carnegie Institute of Washington, Baltimore, MD) to make the plasmid pR48. Green fluorescent protein (GFP) is fused ∼20 amino acids after the S6 transmembrane domain. pR48 (30 ng/μl) and the wild-type *pha-1*-containing plasmid pBX-1 (100 ng/μl) were injected into the germline of *pha-1*;*him-5* hermaphrodites (Mello et al., 1991; Granato et al., 1994).

Twenty mid-L4 males from two transgenic lines were analyzed for *unc-103* expression.

We noted that the fusion protein aggregates mostly on the cytoplasmic membrane of neuronal processes and in many, but not all, cases of cell bodies; however, we detected no deleterious effect of the fusion construct on the behavior of the transgenic animals. In larval hermaphrodites and males we were able to identify some of the cells that had cell body localization of UNC-103::GFP; many neurons express the construct, but because of variability in cell position and cellular localization of the fusion protein in processes, our list of *unc-103*-expressing cells is far from complete. In the head region ALA, ADL, ASK, AVH, AVJ, AIN, AVA, ASJ, SMDD, SIA, ADE, and AVD express the construct. In the tail region PHA, DVC, ALN, and PVP express the construct. The *unc-103* fusion protein accumulates extensively in the processes of many ventral cord neurons; however, we were able to identify only some cells in the AS class of excitatory neurons. In the adult hermaphrodite HSN expresses the *unc-103::gfp* construct.

Injections of pR48 at ≥50 ng/μl were lethal. Some F1 transgenic animals were obtained with 50 ng/μl pR48; however, they did not transmit the extrachromosomal array to subsequent generations. These exceptional F1 animals showed mosaic expression of GFP not only in neurons but also in body wall and pharyngeal muscles (our unpublished observation). Therefore, we cannot rule out that *unc-103* may be expressed in more cell types than neurons, but neurons might tolerate the expression of the fusion construct better than muscles.

Mating observation. We observed mating behavior with a compound microscope fit with a 40× objective. Mating behavior was recorded with an MTI CCD72 black-and-white video camera and a Panasonic AG-6740 time-lapse Super VHS videocassette recorder. We placed five 15–24 hr *unc-38(sy576);egl-19(n582)* adult hermaphrodites and one male on a 5-mm-diameter OP50 lawn made on a 1 cm block of agar. We then placed the agar block on a microscope slide for direct viewing. When the male tail touched the vulva, we started the video recording. Males have difficulty inserting their spicules into young hermaphrodites and will prod the vulva continuously for 10 min or longer. This allowed us to make long recordings of this behavior. To quantify the rate of spicule movements for wild type and *unc-103* null, we slowed the recordings to 1/7 the original speed and manually counted the downstrokes of the spicules.

Results

unc-103 males protract their copulatory spicules independently of mating stimulation

The *C. elegans* male contains two copulatory spicules. Each spicule is attached to two protractor muscles (Fig. 1*A*). When these muscles contract, the attached spicules extrude from the male tail. To understand how specific motor behaviors are regulated during copulation, we screened for mutations that uncouple the spicule insertion step from other mating-specific motor and sensory behaviors. From a screen of 10,000 EMS-mutagenized gametes we isolated the *sy557* allele. Wild-type males keep their spicules extended from their tails only during mating. In contrast, *sy557* males spontaneously protract their spicules in the absence of mating stimulation.

Initially after the L4 molt *sy557* adult males behave normally, but within 1–5 hr they spontaneously protract their spicules (Fig. 1*B–D*). Occasionally, mating-independent protraction is coupled with a dorsal or a ventral tail curl. Affected males sometimes can retract their spicules into their tails; however, their spicules will continue to protract inappropriately. Eventually, the spicules remain permanently protracted.

This mutant phenotype is similar to the protruding spicule phenotype of males that contain the gain-of-function *egl-19(n2368gf)* allele (Lee et al., 1997; Garcia et al., 2001). *egl-19* encodes the *C. elegans* homolog of the L-type voltage-gated calcium channel α1 subunit (Lee et al., 1997). In *egl-19(gf)* males
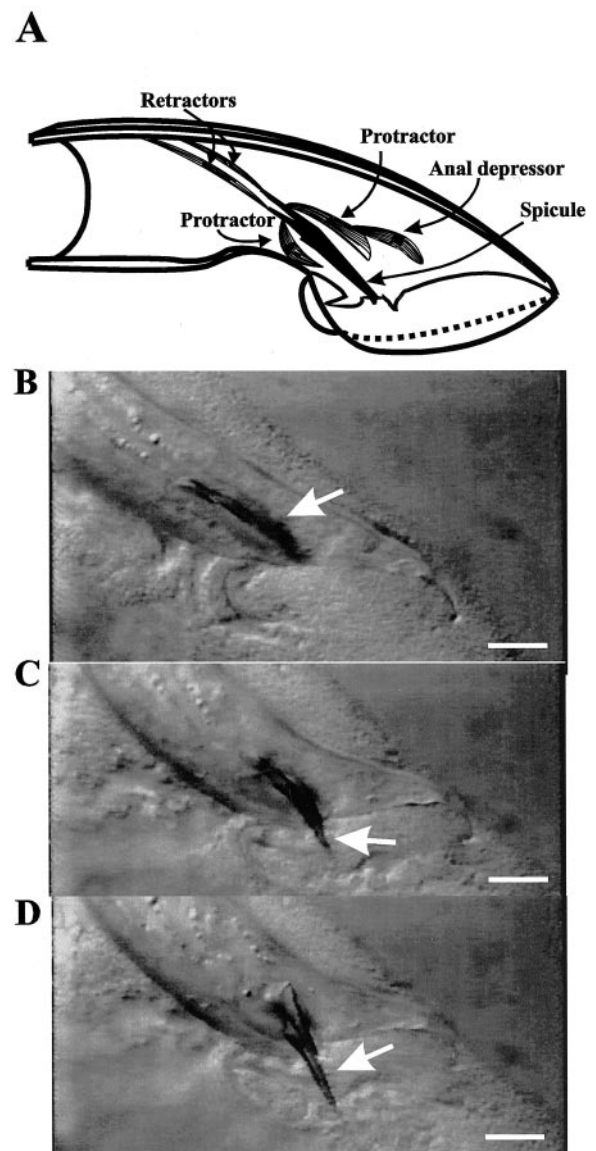

Figure 1. Mating-independent spicule protraction displayed by a *sy557* mutant male. *A* depicts a diagram of the sex muscles that are associated with the spicules (right lateral view of the male tail). The drawing was adapted from Sulston et al. (1980). The spicule is shown in *black*. The dorsal and ventral protractor and retractor muscles, represented as striated cells, are attached to the body wall and the base of the spicule. The anal depressor muscle is attached to the body wall and contacts the dorsal protractor. *B–D* depict individual frames taken from a video recording of a <24 hr virgin *sy557* adult male. The *arrow* points to his copulatory spicules (lateral view). In *B*, the *arrow* points to copulatory spicules that are inside his tail. In *C*, the *arrow* points to the copulatory spicule as it starts to protrude from the tail. In *D*, the spicule is protracted completely. In all panels his anterior is to the *left*, and his dorsal side is to the *top*. Scale bar, 20 μm.

excessive inappropriate channel activity not only may activate protractor contraction prematurely but also subsequently may damage the spicule muscle cells so that the spicules no longer can be retracted. In affected *sy557* males a similar type of defect also might be occurring.

In a population of virgin males kept isolated from hermaphrodites, ~60% of the males will protract their spicules permanently within 24 hr after they emerge from their L4 cuticle (Table 1). Once the spicules remain protracted, the *sy557* male is incapable of siring progeny. Exceptional *sy557* males that do not display mating-independent spicule protraction can sire progeny,

Table 1. *unc-103* mutants display mating-independent spicule protraction

Genotype	Percentage of protracted males[a]	n[b]
Wild type	0	100
unc-103(sy557) H165N, W244R	66	454
+/*unc-103(sy557)*	20	487
unc-103(e1597gf)	0	80
+/*unc-103(e1597)*	0	62
unc-103(sy673, e1597) W85opal	20	312
unc-103(sy670, e1597) G306E	37	81
unc-103(e1597, sy674) P426S	7	46
unc-103(n1213) null	29	324
+/*unc-103(n1213)* null	0	23
unc-103(sy557)/unc-103(n1213)	40	64
unc-103(sy673, e1597)/unc-103(n1213)	13	32
unc-103(sy670, e1597)/unc-103(n1213)	17	78
unc-103(e1597, sy674)/unc-103(n1213)	7	29

[a]Males whose spicules remain permanently protracted.

[b]Number of males assayed.

albeit at an efficiency less than wild-type males (wild-type mating efficiency: 0.74 ± 0.11, mean ± SD, n = 3 trials; *sy557* mating efficiency: 0.29 ± 0.12, n = 2 trials).

sy557 males and hermaphrodites do not show any gross abnormalities in other behaviors such as locomotion, pharyngeal pumping behavior, defecation, or chemotaxis. However, *sy557* hermaphrodites sometimes lay eggs at a slightly higher rate than wild type (our unpublished observation).

We mapped *sy557* to LGIII between *daf-2* and *dpy-17* (see Materials and Methods). Using single nucleotide polymorphisms as mapping markers (Wicks et al., 2001), we narrowed *sy557* to a 140 kb interval between the polymorphisms snp C30D11.4 and snp R10E4.1. We noted that *unc-103*, the *C. elegans* homolog of *Drosophila seizure/erg* and human *h-erg* (Titus et al., 1997; Reiner et al., 1999; J. H. Thomas, personal communication), was in this interval. Loss-of-function mutations in the *Drosophila* K+ channel gene cause general fly hyperactivity and spontaneous seizures (Jackson et al., 1984, 1985; Kasbekar et al., 1987; O'Dowd and Aldrich, 1988; Elkins and Ganetzky, 1990). The phenotypic similarity of spontaneous *C. elegans* male protractor contractions and spontaneous seizures in *Drosophila* suggested that *sy557* might affect the K+ channel gene in *C. elegans*.

We sequenced the predicted coding regions of *unc-103* in our reference wild-type strain and in the *sy557* mutant and found that the *sy557* mutant contains two missense mutations in *unc-103*. The mutations change a histidine at amino acid 165 to an asparagine near the S3 transmembrane region and a tryptophan at position 244 to an arginine in the S5 region. Both of these substitutions are in amino acids that are conserved in the human, fly, and worm protein (Fig. 2).

Reduction of *unc-103* causes spontaneous spicule protractor contraction

To determine how the two missense mutations in *unc-103(sy557)* cause mating-independent spicule protraction, we compared the phenotype induced by *unc-103(sy557)* with the phenotypes of loss-of-function, gain-of-function, and null alleles of *unc-103*. *unc-103(sy557)* is semidominant because heterozygous males protract their spicules (Table 1). To determine whether semidominance was attributable to *sy557* conferring enhanced activity to UNC-103, we first compared *unc-103(sy557)* with the canonical gain-of-function *unc-103(e1597gf)* allele. *unc-103* was defined first by the dominant gain-of-function mutations *e1597*

and *n500* (Hodgkin, 1983; Park and Horvitz, 1986). Animals containing *unc-103(e1597gf)* are extremely lethargic and egg-laying defective in both the homozygous and heterozygous state (Hodgkin, 1983; Park and Horvitz, 1986; Reiner et al., 1995). None of these behavioral defects is seen in *unc-103(sy557)* animals. An additional difference is that *unc-103(e1597gf)* males do not protract their spicules spontaneously (Table 1). Thus the basis for the semidominance of *unc-103(sy557)* is probably not the same as for *unc-103(e1597gf)*.

Work by others has shown that suppression of *unc-103(e1597gf)*-induced lethargy and defective egg-laying behaviors can be caused by intragenic mutations that reduce gene function (Park and Horvitz, 1986). To determine whether *unc-103(sy557)* caused a reduction in UNC-103 activity, we isolated additional intragenic revertants of *unc-103(e1597gf)* and asked whether intragenic mutations that eliminate the *e1597* phenotype also can induce spicule protraction. We first EMS-mutagenized *unc-103(e1597gf)* animals and then selected F2 hermaphrodites that had wild-type locomotion and egg-laying behavior. We then assayed their male progeny for spontaneous spicule protraction. We isolated three intragenic revertants of *unc-103(e1597gf)*: *unc-103(sy673, e1597gf)* W85opal, *unc-103(sy670, e1597gf)* G306E, and *unc-103(e1597gf, sy674)* P426S. *sy673* creates a stop codon that truncates the gain-of-function protein after the first 85 amino acids, which is a few hundred amino acids upstream of the *e1597* lesion (J. H. Thomas, personal communication); *sy670* changes the conserved glycine at amino acid position 306 to a glutamic acid in the ion selectivity filter of the pore region (Doyle et al., 1998); and *sy674* changes a proline at amino acid position 426 to a serine, which is downstream of the *e1597* lesion (J. H. Thomas, personal communication) (Fig. 2).

In all, 20 and 37% of males containing *unc-103(sy673, e1597gf)* W85opal and *unc-103(sy670, e1597gf)* G306E, respectively, displayed seizures of their protractor muscles; in contrast, only 7% of *unc-103(e1597gf, sy674)* P426S showed the phenotype. We believe that both the W85opal and G306E lesions reduce K+ channel function of the gain-of-function protein. The stop mutation truncates the gain-of-function protein within the first transmembrane region, and others have demonstrated that mutating the equivalent glycine in the ion selectivity filter of the human HERG channel disrupts ion conduction (Sanguinetti et al., 1996). We also assayed the effects that an *unc-103* null mutation had on spontaneous protractor contraction. *unc-103(n1213)* was isolated originally as a revertant of *unc-103(e1597gf)* (Park and Horvitz, 1986) and is a deletion of *unc-103* (Reiner et al., 1999; J. H. Thomas, personal communication). Similar to *unc-103(sy673, e1597gf)* W85opal and *unc-103(sy670, e1597gf)* G306E males, 29% of *unc-103(n1213)* null males showed spontaneous protractor muscle contraction (Table 1). Thus we conclude that reduction of UNC-103 activity results in mating-independent spicule protraction. The *unc-103(e1597gf, sy674)* P426S mutation had a small effect on activating premature spicule protraction, suggesting that the *e1597gf, sy674* channel still might have some functional activity. Because *unc-103(e1597gf, sy674)* animals show locomotion and egg-laying behavior similar to wild type, the P426S change might counteract the effects of the *e1597* mutation so that some level of proper channel function is restored.

Although loss-of-function and null mutations in *unc-103* caused mating-independent spicule protraction, the percentages of males showing the defect were lower compared with *unc-103(sy557)* H165N, W244R males. To test whether UNC-103 (H165N, W244R) also might interfere with wild-type UNC-103 function, we analyzed its genetic interaction with the gain-of-

function *unc-103* allele. Arecoline, an ace-tylcholine agonist, can induce the spicule protractor muscles to contract artificially (Garcia et al., 2001). The *unc-103(e1597gf)* mutation reduces the effectiveness of the agonist to induce muscle contraction (Fig. 3*A*). At a concentration in which ~90% of wild-type males respond to the drug, homozygous *unc-103(e1597gf)* males are insensitive, and 30% of *unc-103(e1597gf)/+* males and 38% of *unc-103(e1597gf)/null* males respond (Fig. 3*B*). We put *unc-103(e1597gf)* in trans to the other *unc-103* alleles to determine whether they could interfere with the gain-of-function mutant channel and consequently restore drug sensitivity. When we made comparisons to *unc-103(e1597gf)/null*, we found that putting *unc-103(sy673, e1597gf)* W85opal or *unc-103 (e1597gf, sy674)* P426S in trans to *unc-103(e1597gf)* reduces sensitivity to arecoline. This suggests that for *sy673* translational read-through might be occurring at the opal stop codon so that some level of full-length *e1597* protein is made; for *sy674* the P426S change probably does not suppress the *e1597* lesion completely. In contrast, *unc-103(sy670, e1597gf)* G306E can interfere partially with *unc-103(e1597gf)*; this is not surprising because others have shown that mutating the glycine at that position in the ion selectivity filter confers dominant-negative properties to the protein (Sanguinetti et al., 1996). Additionally, we found that *unc-103(sy557)* H165N, W244R completely interfered with *unc-103(e1597gf)* and restored drug sensitivity to wild-type level, thus suggesting that *unc-103(sy557)* also might act as a dominant-negative protein. However, because mating-independent spicule protraction occurred more frequently in *unc-103(sy557)* than the null, the UNC-103 (H165N, W244R) protein must have, additionally, properties that cause it to interfere with the regulation of spicule protraction behavior.

The SPC motor neurons and the anal depressor muscle facilitate *unc-103(sy557)*-induced spicule protraction

The SPC motor neurons and the anal depressor muscle are attached directly to the protractor muscles (Fig. 4*A*) (Sulston et al., 1980). During mating the SPC motor neurons are essential for triggering prolonged contraction; in contrast, the anal depressor muscle is nonessential for mating behavior under standard conditions (Garcia et al., 2001). We ablated the SPC motor neurons and the anal depressor muscle to determine whether these cells were required for *unc-103(sy557)*-mediated spicule protraction. Ablating either cell type separately did not affect the mutant phenotype; however, when both cells were killed, the spicules did not protract spontaneously (Table 2). This observation suggests that *unc-103(sy557)* does not act solely in the protractor muscles to cause spontaneous spicule protraction. We ablated other neurons in the vicinity of the protractor and anal depressor muscles (Sulston et al., 1980), but unlike the SPC-anal depressor double ablation, killing combinations of other neurons did not eliminate mutation-induced protraction (Table 2).

Because *unc-103(sy557)* does not act solely in the protractor muscles, we fused GFP to *unc-103* sequences to determine which of the cells associated with the spicules express the gene. Our
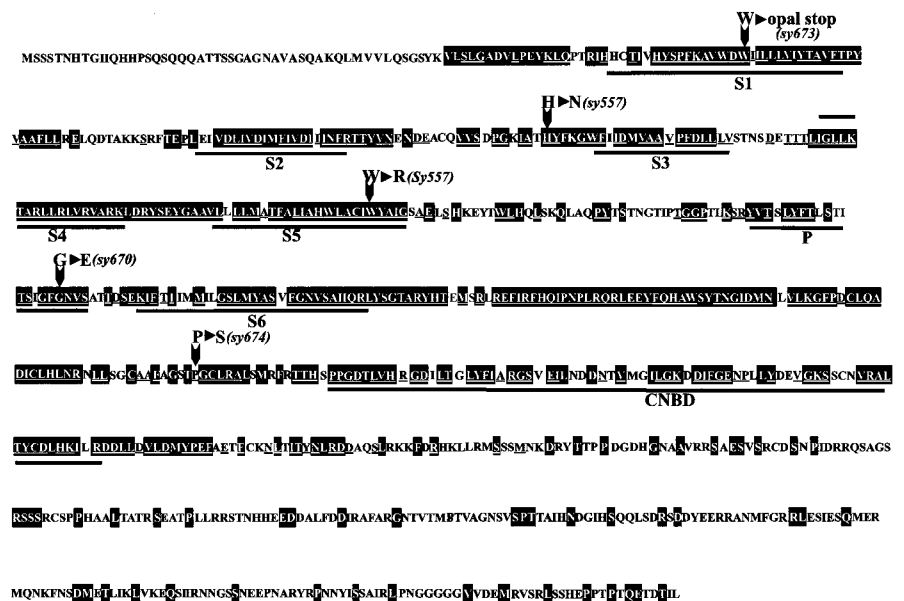

Figure 2. Locations of mutation-induced amino acid changes in UNC-103. Amino acid residues that are identical to human HERG are *boxed*; those that are identical to *Drosophila* ERG are identified by the *thin underline*. Approximate regions of UNC-103 that correspond to the six membrane-spanning (*S1–S6*), the pore (*P*), and the cyclic nucleotide-binding (*CNBD*) domains are identified by the *thick underline*. Downward-pointing arrowheads show amino acids that are changed in the mutant alleles isolated in this study. The amino acid changes are listed next to the mutant allele.

unc-103::GFP construct expressed broadly in the head and ventral cord neurons in both males and hermaphrodites (see Materials and Methods). In the male tail the SPC motor neurons and the PCB postcloacal sensory neurons expressed the construct (Fig. 4*B*). The expression in SPC is consistent with those neurons contributing to *unc-103(sy557)*-induced spicule protraction. Irregular expression was seen in the PCA postcloacal sensory neurons; in other neurons that are associated with spicules, such as the PCC postcloacal sensory neurons and the SPD and SPV spicule sensory neurons, the fusion protein was not expressed.

The *unc-103(sy557)* phenotype requires acetylcholine production and L-type voltage-gated calcium channels

To determine which molecular components are acting inappropriately when *unc-103* is compromised, we surveyed mutations that affect general synaptic transmission and muscle contraction for suppression of the *unc-103(sy557)* phenotype. We found that mutations in *cat-1* [vesicular monoamine transporter (Duerr et al., 1999)], *unc-49* [GABA receptor (Bamber et al., 1999)], *unc-38* [nicotinic acetylcholine receptor subunit (Fleming et al., 1997)], *unc-64* [syntaxin 1A (Ogawa et al., 1998; Saifee et al., 1998)], and *unc-2* [α1 voltage-gated calcium channel subunit (Schafer and Kenyon, 1995)] did not reduce *unc-103(sy557)*-induced protraction. However, mutations in *unc-31* [neurosecretory calcium-binding protein (Ann et al., 1997)], *unc-13* [DAG-binding synaptic transmission component (Ahmed et al., 1992)], and *unc-68* [ryanodine receptor calcium channel (Maryon et al., 1996)] had a very small to moderate effect on reducing spicule protraction. In contrast, mutations in *cha-1* [choline acetyltransferase (Alfonso et al., 1994)] and *egl-19* [L-type α1 voltage-gated calcium channel subunit (Lee et al., 1997)] suppressed the *unc-103(sy557)* phenotype (Table 3). The suppression by *cha-1* and *egl-19* suggests that spontaneous protractor contraction requires L-type voltage-gated calcium channels and acetylcholine (ACh) production. This suppression was expected because EGL-19 is required for prolonged protractor contraction during mating behavior, and
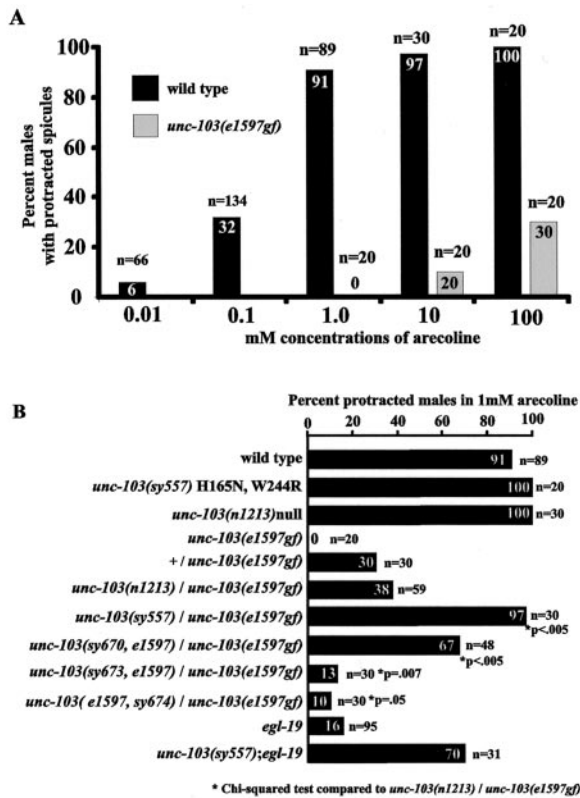
Figure 3. Arecoline-induced spicule protraction in *unc-103* mutants. *A*, The sensitivities of *unc-103(e1597gf)* males to different concentrations of arecoline are compared with wild type. The *numbers* in the columns refer to the percentages of males that protract their spicules with drug exposure; *n* refers to the number of males assayed. *B* shows how different *unc-103* genotypes affect the percentages of males that protract their spicules in 1 mM arecoline.

cholinergic transmission from SPC, PCB, PCC, and additional neurons can cause the anal depressor and protractor muscles to contract (Garcia et al., 2001).

UNC-68 ryanodine receptor calcium channels are not essential for spicule insertion behavior, but they contribute to the frequency of periodic contractions. The *unc-68(r1158)* null allele reduced the *unc-103(sy557)*-induced protraction by one-half, suggesting that, in some animals, calcium efflux from sarcoplasmic stores can contribute to muscle seizures. However, because many *unc-103(sy557)*;*unc-68(r1158)* males still had protracted spicules, intracellular calcium mobilization must not be essential.

Although mutations in *unc-13, unc-31,* and *unc-64* reduce synaptic transmission, they had little effect on the *unc-103(sy557)* phenotype. This finding was unexpected because reducing ACh production with the *cha-1* mutation affected mating-independent spicule protraction. The *unc-13, unc-31,* and *unc-64* alleles used in this work might not reduce synaptic transmission enough to affect spontaneous protraction drastically; alternatively, *unc-103(sy557)* might allow the neurons to bypass the requirement for wild-type action of these three genes.

unc-103 males execute premature prolonged protractor contraction during mating

When wild-type males mate with 24 hr or younger adult hermaphrodites, they have difficulty inserting their spicules into the vulva. The tips of the spicules rapidly prod the vulval slit until eventually they penetrate the vulva. Prodding behavior is facilitated by UNC-68-mediated periodic protractor contractions. When the spicule tips penetrate the vulva, the SPC motor neu-
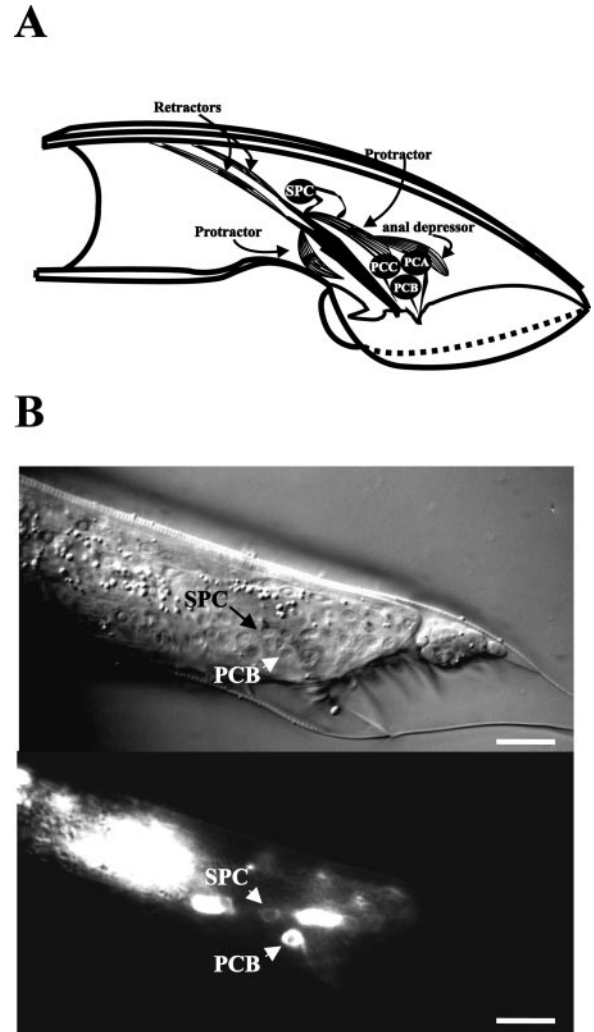

Figure 4. Cells involved in *unc-103(sy557)*-induced spicule protraction. *A* depicts the right lateral view of the male tail, highlighting cells that are associated with the spicules. The drawing was adapted from Sulston et al. (1980). The spicule is shown in *black*. The dorsal and ventral protractor muscles, represented as striated cells, are attached to the body wall and the base of the spicule. The anal depressor muscle is attached to the body wall and contacts the dorsal protractor. The right SPC motor neuron contacts the protractors and the base of the spicule. The right PCA, PCB, and PCC neurons send their sensory processes to the right postcloacal sensillum. *B* depicts Nomarski (*top*) and fluorescence (*bottom*) images of the lateral tail region of a male that expresses the *unc-103::GFP* reporter construct. Scale bars, 20 μm. The male in the image is in L4 lethargus. The PCB postcloacal sensory neuron and the SPC spicule motor neuron, in addition to some unidentified neurons, express the reporter gene.

rons have been proposed to trigger EGL-19-mediated prolonged contraction, causing the spicules to remain extended from the male tail (Garcia et al., 2001).

Although the *unc-103* mutant phenotype suggests that UNC-103 helps to keep the spicule muscles inactive before mating, the protein might function additionally during sex. To address this, we observed how nonprotracted *unc-103* males try to insert their spicules into 24 hr adult hermaphrodites. We observed that, instead of consistently prodding the vulval slit with their spicule tips until penetration, all *unc-103(sy557)* H165N, W244R males (*n* = 2), *unc-103(sy673, e1597gf)* W85opal males (*n* = 10), and *unc-103(n1213)* null males (*n* = 10) repeatedly protract their spicules during prodding behavior. The prematurely extended spicules would depress the hermaphrodite vulva rather than penetrate it. After protraction the spicules retract into the male tail to

Table 2. Ablations of protractor associated cells

Ablations in *unc-103(sy557)* H165N, W244R	Percentage of protracted males[a]	n[b]	p value[e]
Nonoperated	59	75	
P9.p, P10.p, P11.p[c]	83	12	0.11
P(l/r), U(l/r)[d]	54	11	0.76
PCA(l/r), PCB(l/r), PCC(l/r)	88	8	0.12
P9.p, P10.p, P11.p, PCA (l/r), PCB (l/r), anal depressor	57	7	0.9
SPD (1/r), SPV (1/r)	50	8	0.6
DVA, DVB, DVC	43	14	0.25
SPC(l/r)	62	34	0.68
Anal depressor	63	19	0.76
AVL, DVB, SPC (l/r)	30	9	0.14
SPC(l/r), anal depressor	0	20	<0.005

[a]Males whose spicules remain permanently protracted.

[b]Number of males assayed.

[c]Ablations remove PGA, PDC, PVY, PVV, PVZ, HOA, HOB, and associated structural cells.

[d]l/r refers to left and right cell. Ablations remove EF(1/2, 3/4) and DX(1/2, 3/4); males have crumpled spicules that still protract.

[e]χ^2 test compared with nonoperated *sy557*.

Table 3. Mutant genes that affect *unc-103(sy557)*-induced protraction

Genotype	Percentage of protracted males[b]	n[c]	p value[d]
Wild type[a]	0	100	
unc-103(sy557) H165N, W244R[1]	66	454	
unc-38;unc-103(sy557)	83	59	n.d.
unc-103(sy557);unc-49	72	53	n.d.
unc-103(sy557);unc-64	70	148	n.d.
unc-103(sy557);cat-1	60	27	0.2
unc-103(sy557);unc-2	54	28	0.18
unc-103(sy557);unc-31	52	85	0.02
unc-13; unc-103(sy557)	47	43	0.02
unc-103(sy557);unc-68	31	45	<0.005
unc-103(sy557);cha-1	11	63	<0.005
unc-103(sy557);egl-19	5	60	<0.005

n.d, Not done.

[a]Values taken from Table 1.

[b]Males whose spicules remain permanently protracted.

[c]Number of males assayed.

[d]χ^2 compared with *sy557*.

resume prodding behavior. We ablated the SPC motor neurons in five *unc-103(n1213)* null animals and observed their spicule insertion behavior. All of the operated males prematurely protracted their spicules during prodding behavior, demonstrating that the SPC motor neuron is not essential for this abnormal behavior.

We analyzed four intact *unc-103(n1213)* null males and four wild-type males in greater detail by counting the movements of their spicules. Video recordings of spicule insertion behavior were played back at slower speeds to determine the number of spicule movements during prodding behavior and the frequency of spontaneous protraction (Fig. 5). In contrast to the steady rhythmic prodding of wild-type spicules, we found that the spicules of *unc-103(n1213)* null males arrhythmically prodded the vulval slit 1–9 times/sec. We also noted that complete spicule protraction punctuated every 1–12 sec of prodding behavior. Thus we conclude that *unc-103* plays at least two roles in the male: to suppress protractor muscle contractions until proper mating signals are sensed and to regulate periodic and prolonged contractions during mating.

Discussion

UNC-103 keeps the spicule protractor muscles inactive before mating

Male mating behavior consists of many stereotyped sub-behaviors that must be coordinated to insure efficient mating. To impregnate a hermaphrodite, the male moves backward along her body scanning for the vulva. If the male fails to locate the vulva on one side of the hermaphrodite, he will turn to her other side and resume vulva location behavior. When he locates her genitalia, he stops backward locomotion and adjusts the position of his tail over the vulva so that the spicule tips contact the vulval slit. The neurons used for vulva location behavior also signal the spicule protractor muscles to contract and relax rhythmically until the spicules penetrate the vulval barrier. When the spicules breach the vulva, the SPC motor neurons signal the protractor muscles to remain contracted until sperm transfer is completed.

To understand how different spicule muscle behaviors are regulated, we isolated mutations that cause males to protract their spicules in the absence of mating stimulation. From 10,000 mutagenized gametes we isolated *unc-103(sy557)*. *unc-103(sy557)* causes ~60% of virgin males to protract their copulatory spicules within 24 hr of adulthood. UNC-103 encodes a putative voltage-gated K$^+$ channel that is homologous to human *h-erg* and *Drosophila seizure/erg*. The *sy557* lesion contains two mutations that create an H165N and W244R amino acid change in the K$^+$ channel protein. Null and loss-of-function alleles of *unc-103* also induce spontaneous spicule protraction, suggesting that before mating behavior UNC-103 keeps the spicule muscles from contracting. No other gross behavioral abnormalities are seen in *unc-103*(null) and *unc-103(sy557)* males, suggesting that cells in the spicule insertion behavioral circuit are more sensitive to changes in this K$^+$ channel than other *unc-103*-expressing cells.

UNC-103 may act in parallel with other proteins to regulate protractor muscle activity

Although *unc-103(sy557)* and the other *unc-103* alleles cause mating-independent spicule protraction, males that do not protract their spicules within 24 hr of adulthood will not display the abnormal phenotype later. Two scenarios can account for why some males do not show the defect: (1) buffering systems that act parallel to *unc-103* can overcompensate for loss of *unc-103* function or (2) unaffected males are not exposed to some environmental stimuli that normally activates an *unc-103*-regulated excitatory pathway.

We favor the hypothesis that, at some efficiency, compensating mechanisms can attenuate spontaneous protractor activity in these exceptional males. The *sy557* allele induces mating-independent spicule protraction at a higher percentage than compared with other *unc-103* alleles. This difference could be attributable to the *sy557* protein interfering with these hypothetical compensating mechanisms. In our mutant screen we identified three additional mutations that cause premature spicule protraction similar to *unc-103(sy557)*. These mutations are not in *unc-103* and might affect components that act parallel to the K$^+$ channel (our unpublished data).

Behavioral and pharmacological properties of *unc-103(sy557)*/+ and *unc-103(sy557)*/*unc-103(gf)* males suggest that *unc-103(sy557)* encodes a dominant-negative protein that also may interfere promiscuously with other proteins. The H165N and W244R changes in the *sy557* protein affect amino acids that are conserved in the human, fly, and worm protein. The hydrophobic aromatic to a hydrophilic basic side chain change at position 244 in the S5 transmembrane domain of *unc-103* may disrupt
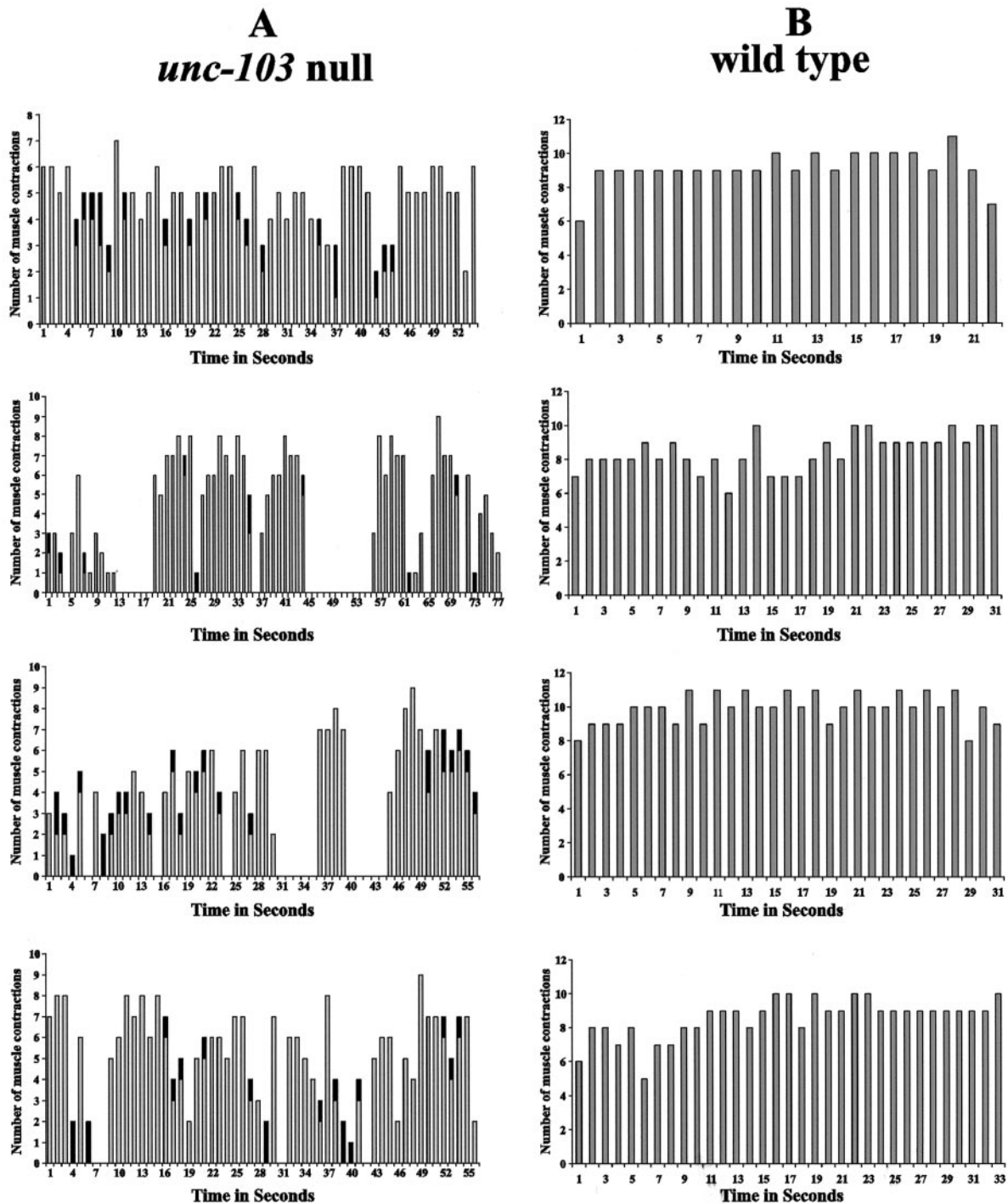
Figure 5. Number of muscle contractions per second during prodding behavior. *A* shows the profiles of muscle contractions of four *unc-103(n1213)* males. The *horizontal axis* displays sequential seconds during the window of observation. *Gray columns* represent the number of spicule movements during each second of prodding behavior. The *black* in some of the *gray columns* refers to a prolonged contraction that occurred during that time interval. Time intervals that show no *gray columns* are attributable to the hermaphrodites shifting their position during the observation period. Males temporarily stop prodding behavior to reorient their tails over the vulva. *B* shows the profiles of muscle contractions of four wild-type males.

proper channel function. However, the consequence of the H165N substitution near the S3 transmembrane region is not obvious. UNC-103 shares ~40% amino acid identity to the *ether-a-go-go*-encoded (*eag*) K⁺ channels in humans, flies, and worms (Titus et al., 1997; Weinshenker et al., 1999). Interestingly, among the *eag* proteins asparagine is the conserved amino acid at that equivalent amino acid position near the S3 transmembrane region. We speculate that the *sy557*-encoded H165N substitution might cause *unc-103* to interact with other K⁺ channels artificially.

Neuronal ACh input facilitates *unc-103(sy557)*-induced spicule protraction

Ablation of both the SPC cholinergic neuron and the anal depressor muscle is required to suppress spontaneous spicule protraction. Removing either one separately has little effect, suggesting that both cells are capable of triggering the protractor muscles to sustain contraction. During mating behavior only the SPC motor neurons are essential for prolonged contraction. However, under artificial conditions neither SPC nor the anal depressor is re-

quired because ACh agonists can activate protractor contraction directly even when both cell types are ablated (Garcia et al., 2001).

unc-103 is expressed in the SPC neurons, consistent with the contribution of those cells to the mutant phenotype. In hermaphrodites and larval males the AVL and DVB GABAergic neurons activate anal depressor muscle contractions (McIntire et al., 1993). In adult males these neurons might not contribute actively to *unc-103(sy557)*-induced protraction because killing these cells does not eliminate the mutant phenotype. The anal depressor muscle may have other inputs that facilitate spicule protraction in the adult male. We do not see *unc-103* expression in the anal depressor, protractor, or any other type of muscle cell in the stable transgenic lines used in this study; however, because of caveats of using extrachromosomal arrays in *C. elegans*, we cannot rule out that *unc-103* also might express in muscles (see Materials and Methods).

Similar to *unc-103(sy557)*, aldicarb, an acetylcholine esterase inhibitor, can induce males to protract their spicules. Ablation of the SPC cholinergic motor neurons and the anal depressor muscle can attenuate aldicarb-induced protraction, demonstrating that ACh can act on or via these cells to induce the protractors to contract. Mutations in *cha-1* choline acetyltransferase or *unc-64* syntaxin also reduce aldicarb-induced protraction. Thus in the absence of mating stimulation low levels of cholinergic transmission to the protractor and anal depressor muscle occur (Garcia et al., 2001). In contrast to aldicarb-induced protraction, the *unc-103(sy557)* phenotype is reduced by the *cha-1* mutation, but not by an *unc-64* mutation.

Worms containing *unc-64* syntaxin mutations are severely compromised for locomotion. Elimination of SLO-1 calcium-activated, voltage-gated K$^+$ channels suppresses *unc-64*-induced lethargy. This observation suggests that *unc-64* mutants express enough syntaxin proteins to promote locomotion, but not enough to overcome negative regulation of synaptic transmission by SLO-1 channels in ventral cord neurons (Wang et al., 2001). In cholinergic neurons that are involved specifically in spicule insertion behavior, UNC-103 in conjunction with SLO-1 and other K$^+$ channels might participate in attenuating UNC-64-mediated synaptic transmission. We speculate that *unc-103(sy557)* might suppress *unc-64* synaptic transmission defects in mating-specific cells to induce spicule protraction.

UNC-103 regulates prolonged contraction during mating

During mating behavior ACh initiates two types of protractor muscle contractions that use different calcium channels. UNC-68 ryanodine receptor calcium channels are used during periodic contractions, and EGL-19 L-type voltage-gated calcium channels are needed for prolonged contraction. These calcium channels also are differentially required for levamisole-induced (an ACh agonist) and arecoline-induced spicule protraction. The difference between levamisole and arecoline activity is that the former requires UNC-68, whereas the latter requires EGL-19 (Garcia et al., 2001). We find that spicule protraction induced by *unc-103(sy557)* is similar to arecoline because *egl-19* can suppress the *unc-103(sy557)* phenotype greater than *unc-68*. However, the *unc-103* allele also partially can restore arecoline sensitivity to *egl-19* males (Fig. 3B). We speculate that UNC-103 may regulate a signal transduction pathway that can be stimulated by arecoline, requires L-type voltage-gated calcium channels, and is used to prolong muscle contraction.

unc-103 mutants that do not protract their spicules permanently show a consistent abnormal spicule insertion behavior during mating. As *unc-103* mutant males try to breach the vulva,

the rhythm of periodic protractor contractions is abnormal and is disrupted by spurious prolonged contraction. In contrast to wild type, these premature prolonged contractions do not require the SPC motor neurons. Considering that the protractor muscles must distinguish between which types of cholinergic-induced contraction to use during spicule insertion behavior, UNC-103 might suppress EGL-19-used cholinergic pathways while the protractor muscles are undergoing ACh-stimulated periodic contractions.

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