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Regulation of Distinct Muscle Behaviors Controls the *C. elegans* Male's Copulatory Spicules during Mating

L. René Garcia, Pinky Mehta, and Paul W. Sternberg[1]
Howard Hughes Medical Institute
Division of Biology
California Institute of Technology
Pasadena, California 91125

Summary

We demonstrate through cell ablation, molecular genetic, and pharmacological approaches that during *C. elegans* male mating behavior, the male inserts his copulatory spicules into the hermaphrodite by regulating periodic and prolonged spicule muscle contractions. Distinct cholinergic neurons use different ACh receptors and calcium channels in the spicule muscles to mediate these contractile behaviors. The PCB and PCC sensory neurons facilitate periodic contraction through muscle-encoded UNC-68 ryanodine receptor calcium channels. The SPC motor neurons trigger prolonged contraction through EGL-19 L-type voltage-gated calcium channels. The male gonad then lengthens the duration of EGL-19-mediated prolonged muscle contraction. This regulation of muscle contraction provides a paradigm to explain how animals initiate, monitor, and maintain a behavioral motor program.

Introduction

In higher animals, neurons sense and integrate multiple environmental signals, and muscles translate the neuronal secretions into a physical output. A simple motor unit can consist of a sensory neuron that communicates to a muscle cell. Complex behavioral algorithms can then be built by compiling these simple units. By dissecting complex behaviors encoded within simple nervous systems, one can understand the design principles underlying behavioral algorithms.

Caenorhabditis elegans male mating is a complex behavior amenable for intense analysis. The *C. elegans* male has a small nervous system, and at least 79 of his 381 neurons facilitate mating (Sulston et al., 1980; Loer and Kenyon, 1993; Liu and Sternberg, 1995). The stereotyped mating behavior is initiated when the male tail contacts the hermaphrodite. The male presses against the hermaphrodite and moves backward searching for the vulva. If the initial scan fails to locate the vulva, he turns to the other side of his mate and continues scanning. When the male contacts the vulva, he stops backward locomotion, inserts his copulatory spicules, and transfers sperm (Chalfie and White, 1988). Although male mating is complicated, it can be simplified into steps that allow dissection (Liu and Sternberg, 1995).

We focus here on a mating step performed in the sexual behavior of many organisms, i.e., the penetration of a male's copulatory organ into his mate's vulva. The *C. elegans* male inserts two copulatory spicules into the hermaphrodite's vulva to lock onto his mate and to aid in sperm transfer. Both spicules consist of a sclerotized cuticle shell that encases the dendrites of the SPD and SPV sensory neurons (Figure 1; Sulston et al., 1980). Five muscles are attached to each spicule. Two retractor muscles (retractors) hold each spicule inside the male tail, and two protractor muscles (protractors) force each spicule out of the male cloaca during mating. During L4 development, the anal depressor muscle (Anal Dep) reorganizes itself and forms an auxiliary extension to both the left and right protractors. Each protractor set is innervated by an SPC motor neuron (Figure 1; Sulston et al., 1980).

During sex, spicule insertion occurs after the hook sensillum and the postcloacal sensilla (p.c.s.), sensory organs located at the male cloaca (Figure 1), detect the vulva and signal the male to stop backward movement. The hook sensillum is made up of two neurons, HOA and HOB, and the p.c.s. are composed of a bilateral pair of three neurons, PCA, PCB, and PCC. The functions of these two sensory organs are partially redundant: males missing either the hook sensillum or the p.c.s. can sense the vulva, albeit at low efficiency (Sulston et al., 1980; Sulston and White, 1980; Liu and Sternberg, 1995). After the male tail contacts the vulva, the protractors contract, and the attached spicules protrude out the cloaca and enter the vulva.

Spicule insertion is a simple reflex, and the male nervous system must monitor various factors to execute this task. The nervous system computes when to initiate insertion and monitors whether insertion was successful. If insertion fails, the circuitry must reinitiate the behavior. If penetration is successful, the circuitry sustains insertion while the next behavioral step proceeds. Here we address the basis for these decisions by dissecting spicule insertion at multiple levels. At the behavioral and cellular level, we define the contractile behaviors of the protractors and the cells that control them. At the genetic and molecular levels, we determine the neurotransmitter used to activate muscle contraction and genes that promote the activity of the neurotransmitter. Finally, we return to the behavioral level to address the roles that these genes play in regulating the protractors' distinct contractile behaviors.

Results

Two Modes of Spicule-Insertion Behavior

To ask how the spicule muscles function during mating, we quantified the movements of the spicules during insertion behavior. We observed that after the male's cloaca contacted the vulva, the protractors contracted and relaxed repeatedly, which caused the spicules to prod the vulval slit at an average frequency of 7.2 $\pm$ 1.3 Hz (n = 5 males; Table 1). When the spicules penetrated the vulval slit, the protractors dramatically shortened while the retractor muscles lengthened, thereby allowing the spicules to extend through the vulva. After the

[1] Correspondence: pws@caltech.edu

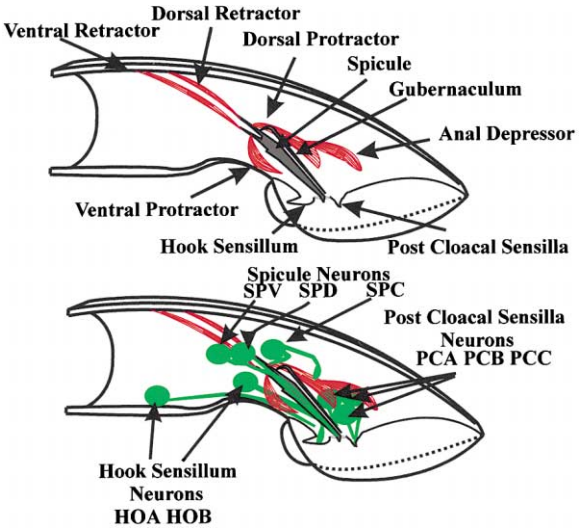

Figure 1. Anatomy of the Spicules

Representations of muscles and neurons discussed in this report. Adapted from Sulston et al. (1980). Cutaway view of the right half of the male tail. The muscles are represented in red, the sclerotized right spicule is in gray, and neurons and their processes are in green. The right retractor and protractor muscles are attached to the body wall and contact the base of the spicule. The anal depressor muscle is attached to the body wall and contacts the dorsal protractor. The sensory processes of the right SPV and SPD neurons are encased by the spicule and are exposed at the spicule tip. Processes of the right SPC motor neuron are associated with the protractors and the base of the spicule. HOA and HOB sensory neurons send their sensory processes to the sclerotized hook. The right PCA, PCB, and PCC neurons send their sensory processes to right postcloacal sensillum.

spicules inserted, the protractors remained contracted until sperm transfer was completed. Thus, spicule insertion can be subdivided into two motor behaviors: a rapid prodding of the spicules at the vulval slit, and a prolonged insertion of the spicules through the vulva.

Spicule insertion is difficult for males to accomplish (Liu and Sternberg, 1995), and in most matings, males prod the vulva rather than inserting their spicules. To determine if the switch from periodic to prolonged protractor contraction was triggered by partial vulval insertion, we measured the duration that males prod the vulvae of paralyzed (*unc-31*) hermaphrodites of various ages, naively reasoning that as hermaphrodites lay eggs over time, their vulvae might become more stretched out and thus easier for males to penetrate. Indeed, we observed that males breached the vulvae of older hermaphrodites more efficiently than those of younger hermaphrodites (Figure 2A), although we concede that extended egg-laying may not be the basis of this phenomenon. Males prodded the vulvae of 24-hr-old hermaphrodite adults on average >10 min before penetration. In contrast, males prodded the vulvae of 48, 72, and 96 hr hermaphrodites on average 46 ± 63, 16 ± 17, and 13 ± 24 s, respectively, prior to penetration. The efficiency of switching from periodic to prolonged contraction is similarly affected by the age of wild-type hermaphrodites (Figure 2B). Males prodded the vulvae of 24 hr wild-type adults on average >5 min before penetration, whereas males prodded the vulvae of 72 hr hermaphrodites on average 6 ± 5 s. We thus infer that vulval penetration triggers the spicule muscles to switch from undergoing periodic to prolonged contraction.

Vulva Location Sensory Neurons Initiate Prodding

We observed that prodding occurred only when the male cloaca was in contact with the vulva. The cloacal region contains three sensory organs: the spicule tips, the hook sensillum, and the postcloacal sensilla (p.c.s.) (Sulston et al., 1980). We ablated the neurons in these structures to determine which sensilla initiated periodic protractor contractions.

The p.c.s. and hook neurons signal the male to stop backward movement upon vulva contact. Males lacking the p.c.s. neurons easily lose contact with the vulva, whereas males lacking their hook neurons pass the vulva and prod random areas of the hermaphrodite with their spicules (Liu and Sternberg, 1995). In addition to recognizing the vulva, we observe that the p.c.s. and the hook sensillum independently activate prodding behavior. Removal of both the hook sensillum and the p.c.s. neurons PCA(L/R) and PCB(L/R) (n = 4 males) resulted in males that showed no spicule activity when their tails passed over the vulva or at any other time during mating. However, when we ablated all p.c.s. neurons (n = 9 males) or the hook sensillum (n = 10 males) separately, the hook neurons in all the p.c.s.-ablated males initiated brief prodding upon transient vulva contact, and the p.c.s. in all the hook-ablated males induced the spicules to prod random areas of hermaphrodite cuticle. We conclude that both sensory systems can trigger prodding behavior.

Previous work determined that SPV regulated the timing of sperm transfer and SPD modulated spicule-insertion behavior (Liu and Sternberg, 1995). We observed that neither cell is essential for prodding behavior. Ablation of SPD and SPV neurons (n = 14 males) at early L4 stage or damage to their sensory endings via laser cauterization of the spicule tips at adult stage (n = 10 animals) did not interfere with males' ability to prod or insert their spicules into hermaphrodites.

SPC Motor Neurons Induce Full Spicule Penetration

The SPC neurons innervate the protractors (Sulston et al., 1980) and are required for spicule insertion (Liu and Sternberg, 1995). To determine their role in spicule insertion, we observed the effects of ablating these neurons on periodic and prolonged protractor contraction. During prodding behavior, the protractors of the SPC-ablated males twitched at an average frequency of 5.1 ± 1.1 Hz (n = 5 males), slightly slower than intact males (p = 0.03; Table 1). When SPC-ablated males (n = 10) were mated to easily penetrable 96 hr *unc-31* adult hermaphrodites, they did not extend their spicules through the vulva, but continuously prodded the vulval slit. Therefore, SPC neurons are required for prolonged protractor contraction but not for initiating or sustaining periodic contractions.

Although each SPC neuron is associated with a separate spicule, either cell facilitates insertion of both copu-

Table 1. Rate of Spicule Twitches during Prodding Behavior

	Male[a]	Seconds[b]	Twitches/Second[c]
Wild-type	1	55	6.1 ± 1.4
	2	58	7.8 ± 1.4
	3	59	6.1 ± 1.3
	4	58	7.1 ± 1.0
	5	61	9.1 ± 1.8
SPC neuron ablated	1	39	3.7 ± 1.2
	2	58	5.9 ± 1.5
	3	59	4.4 ± 1.4
	4	15	5.0 ± 1.6
	5	21	6.2 ± 1.3
egl-19(n582)	1	38	3.1 ± 1.0
	2	32	5.2 ± 1.9
	3	34	4.0 ± 1.1
	4	23	5.7 ± 1.7
	5	28	6.8 ± 1.4
unc-68	1	85	0.48 ± 0.31
	2	87	0.68 ± 0.40
	3	44	0.38 ± 0.11
	4	100	0.39 ± 0.11
unc-68; syEx475[pmyo-3::unc-68]	1	29	6.9 ± 1.8
	2	16	8.4 ± 2.4
	3	25	6.9 ± 1.6
	4	25	7.6 ± 0.7
egl-19(st556); syEx515[egl-19(+)]	1	10	3.3 ± 1.1
	2	4	3.8 ± 1.3
	3	8	5.0 ± 0.9
	4	10	6.6 ± 1.4
	5	10	5.3 ± 1.1
egl-19(st556); syEx515[egl-19(+)] M mosaic loss, Anal Dep not properly reorganized	1	30	4.1 ± 1.2
egl-19(st556); syEx515[egl-19(+)] M mosaic loss, Anal Dep ablated	1	15	2.9 ± 0.5
	2	15	3.7 ± 0.7
	3	3	2.7 ± 0.5

[a] Numerical designation for the observed male.
[b] The duration of spicule prodding analyzed.
[c] Mean and standard deviation.

latory structures. When the left SPC neuron was ablated at early L4 stage (n = 25 males), the operated males still inserted both spicules during mating. Therefore, the insertion signal released from a single SPC neuron must have sufficient range to modulate contractile behaviors of both muscle sets, or the muscle sets must transduce the SPC signal between each other.

We ablated combinations of other preanal and dorsal rectal motor and/or interneurons in the male tail to ask if they contribute to prodding or sustained spicule-insertion behavior (see Experimental Procedures). From our cursory survey, only ablations of the hook, p.c.s., and SPC neurons show an obvious spicule-insertion defect. Ablations of other cells mainly affect movement coordination during mating; however, we do not rule out that these neurons modulate spicule-insertion behavior under more natural conditions.

The Male Gonad Sustains Prolonged Protractor Contraction

We observed that after spicule penetration, the protractors remained contracted for 75 ± 20 s (n = 19 males), sufficient time to transfer sperm. To ask if the duration of prolonged contraction was solely determined by the SPC neurons or was modified by ejaculation, we ablated all or part of the male gonad and measured how the duration of protractor contraction was affected.

The male gonad is derived from four precursor cells present at hatching. The seminal vesicle and vas deferens are derived from Z1 and Z4, and the germline is derived from Z2 and Z3 (Kimble and Hirsh, 1979; Kimble and White, 1981; Kimble, 1981). Ablation of the gonad primordium in L1 males resulted in adult males that inserted their spicules; however, the protractors remained contracted for 32 ± 8 s (n = 18 males), much shorter than intact males ($p < 0.0001$). Thus, in addition to the SPC neurons, gonadal factors lengthen the protractor contraction/relaxation cycle.

Ablation of the germline precursor cells Z2 and Z3 in L1 males resulted in adults that lack a germline. After complete spicule insertion and transfer of spermless seminal fluid, the protractors of germline-ablated males stayed contracted for 59 ± 11 s (n = 14 males), longer than gonad-ablated males ($p < 0.0001$) but slightly shorter than intact males ($p = 0.006$). Thus, somatic cells of the gonad can lengthen the duration of protraction contraction.

Ablation of the linker cell (which guides the proximal gonad to the preanal region (Kimble and Hirsh, 1979; Sulston et al., 1980) in early-L4 males results in adults whose vas deferens is not connected to the cloaca.

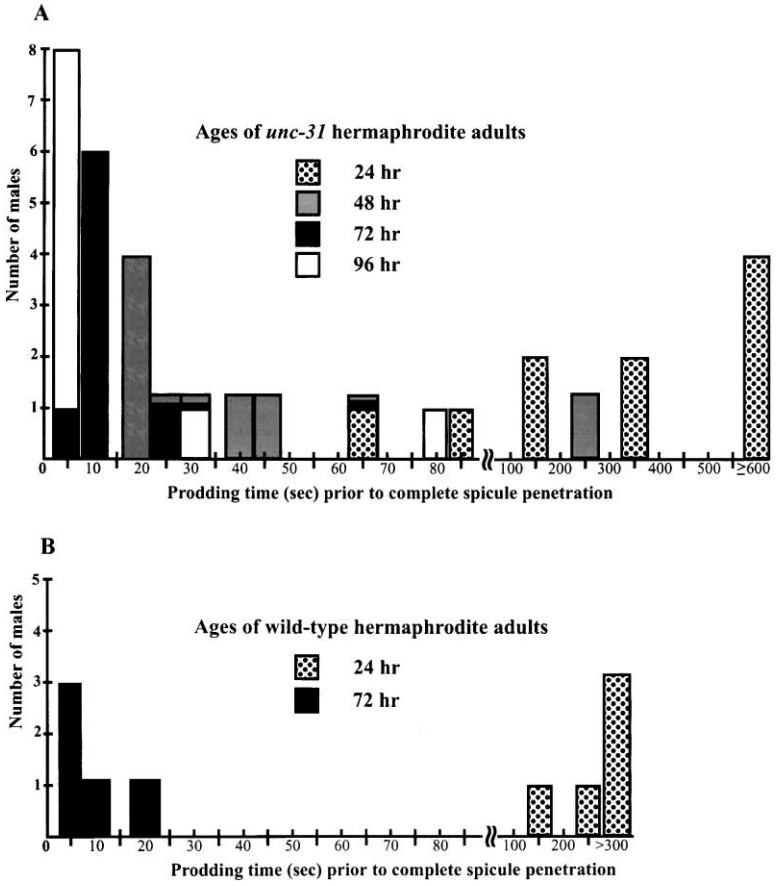

Figure 2. The Duration Virgin Males Prod the Vulva prior to Complete Spicule Insertion

Numbers on the *x* axis represent the upper value in the measured time interval. The scale is demarked every 5 s from 0 to 85 s; after 100 s, the scale is demarked every 50 s.

(A) Ten males were tested for each *unc-31* hermaphrodite age class. The individual times (in seconds) that males prod before they insert their spicules are 150, >600, >600, 107, >600, >600, 322, 319, 66, and 81 for 24 hr hermaphrodite matings; 219, 42, 37, 29, 63, 10, 25, 8, 20, and 9 for 48 hr hermaphrodite matings; 10, 61, 6, 9, 4, 9, 8, 21, 27, and 6 for 72 hr hermaphrodite matings; and 5, 2, 2, 5, 3, 78, 1, 2, 26, and 1 for 96 hr hermaphrodite matings.

(B) Five males were tested for each wild-type age class. The individual times (in seconds) that males prod before they insert their spicules are 277, >300, >300, 139, and >300 for 24 hr hermaphrodite matings and 1, 9, 2, 2, and 16 for 72 hr hermaphrodite matings.

During mating, the operated males inserted their spicules and triggered sperm release from the seminal vesicle; however, the sperm stayed trapped in the male body cavity. In contrast to intact and germline-ablated animals, the spicules of linker cell-ablated males remained inserted for 33 ± 11 s (n = 14 males), similar to gonad-ablated males. Thus, maximal duration of protractor contraction requires proper connection of the gonad to the cloaca.

Acetylcholine Induces Protractor Contraction

We tested common neurotransmitters for the ability to stimulate protractor contraction. We found that the acetylcholine (ACh) agonists levamisole (LEV), nicotine (NIC), and arecoline (ARE) induced protractor contraction when males were bathed in the drugs. The effective concentrations that induced prolonged protraction for 90% of males (EC_{90}) were 2 μM, 258 μM, and 1 mM for LEV, NIC, and ARE, respectively (Table 2). In contrast, GABA, serotonin, and octopamine did not induce the behavior (data not shown).

Since ACh agonists caused the protractors to contract, we asked if endogenous ACh also stimulated these muscles. Aldicarb, an inhibitor of acetylcholine esterase, potentiates endogenous ACh signaling and causes general tonic muscle contraction in worms (Rand and Russell, 1985). We observed this aldicarb-induced protraction (Figure 3A) and the fact that males bathed in 5 mM aldicarb protracted their spicules on average 217 ± 176 s after exposure. In general, aldicarb-induced behaviors require genes involved in synaptic transmission of ACh (Miller et al., 1996a). Indeed, males mutant in *unc-64*, which encodes the *C. elegans* ortholog of syntaxin 1A (Ogawa et al., 1998; Saifee et al., 1998), or in *cha-1*, which encodes choline acetyltransferase (Alfonso et al., 1994), were defective for aldicarb-induced protraction; 55% of *unc-64(e246)* and 72% of *cha-1(p1152)* males required >900 s to protract their spicules (Figure 3A). Thus, neuronal secretion of ACh activates the protractors.

ACh Secretion from PCB, PCC, and the SPC Neurons Induces Protraction

To ask which neurons induced protraction via ACh, we determined the cells in the male tail that express *unc-17,* which encodes a vesicular acetylcholine transporter (Alfonso et al., 1993), and we ablated those cells to determine if they contributed to aldicarb-induced protraction. We found that PCB, PCC, and SPC neurons expressed an *unc-17::gfp* construct beginning at mid-L4 and continuing throughout adulthood (Figure 4). Since these cells are used in insertion behavior, we tested whether they used ACh to activate the protractors.

Ablations of PCB, PCC, and SPC neurons caused males to delay protraction in aldicarb (Figure 3B); 75% of PCB-, PCC-, SPC-ablated males required >300 s to protract. Ablating both the Anal Dep (the protractor accessory muscle) and the neurons caused a similar

Table 2. Drug Concentrations that Cause Spicule Protraction in 90% of Males

Genotype	EC_{90}		
	Levamisole[a]	Arecoline[b]	Nicotine[c]
Wild-type	2 μM	1 mM	258 μM
unc-38	>1 mM	n.d.	n.d.
unc-29	>1 mM	n.d.	n.d.
unc-38; *unc-29*	>1 mM	500 μM	338 μM
unc-38; *egl-30*	n.d.	>10 mM	>6 mM
egl-30	35 μM	1 mM	489 μM
egl-19(n582)	3.7 μM	>10 mM	1.4 mM
unc-68	>1 mM	2 mM	>6 mM
unc-38; syEx469[pmyo-3::unc-38]	20 μM	n.d.	n.d.
unc-68; syEx475[pmyo-3::unc-68]	15 μM	n.d.	101 μM
egl-19(n582); syEx465[pmyo-3::egl-19]	n.d.	1 mM	567 μM

For each concentration, 20–100 males were tested.
[a] Seven concentrations between 100 nM and 1 mM were tested.
[b] Five concentrations between 10 μM and 10 mM were tested.
[c] Five concentrations between 1 μM and 6 mM were tested.

percentage of males, 78%, to require >300 s to protract. However, from those that required >300 s to protract, 40% of the quadruple-ablated males required >900 s to protract (Figure 3B). Therefore, the Anal Dep also contributes to ACh-induced protraction of the spicules. Ablation of each cell type singly (Figure 3C) or doubly (the SPC pair and the Anal Dep) (Figure 3D) had measurable but slight effects as compared to intact, triple-, or quadruple-ablated animals. To rule out the possibility that the quadruple ablations indirectly perturb muscle development and thus cause insensitivity to aldicarb, we ablated PCB, PCC, SPC, and the Anal Dep in mutant males with constitutively protracted spicules to ask if the operation suppresses protractor contraction. *egl-19(n2368sd)* males have hypercontracted muscles (Lee et al., 1997), and this gain-of-function mutation in the

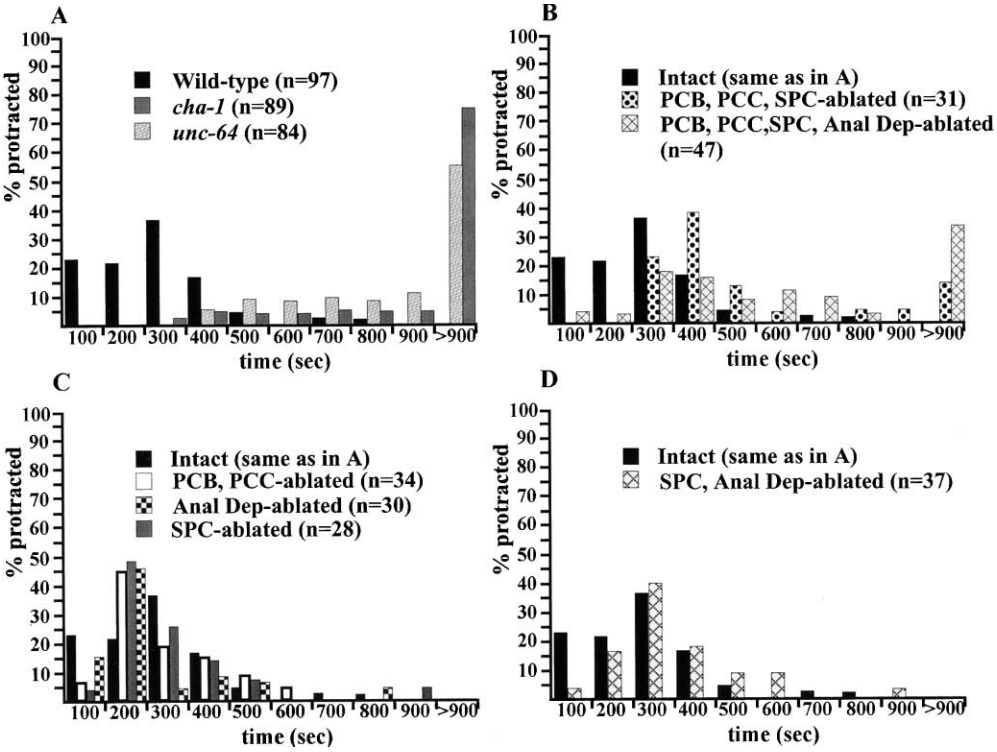

Figure 3. Aldicarb Induces Protraction via the SPC, PCB, PCC Neurons and Anal Depressor

Hash marks on the *x* axis demark intervals of time post drug exposure. Numbers on the *x* axis represent the upper value of percentage spicule protraction in the measured time interval. Colored bars represent the percentage of males that protract their spicules within the time interval.
(A) Aldicarb sensitivity of wild-type, *cha-1*, and *unc-64* males.
(B) Aldicarb sensitivity of wild-type, triple-, and quadruple-ablated males.
(C) Aldicarb sensitivity of wild-type, p.c.s., Anal Dep, and SPC singly-ablated males.
(D) Aldicarb sensitivity of wild-type and SPC and Anal Dep doubly-ablated males.

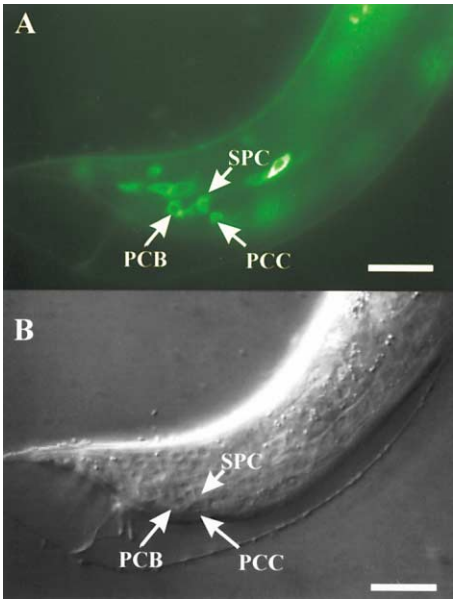

Figure 4. *unc-17* Expression Pattern in the Male Tail

Fluorescence (A) and Nomarski (B) images of the right lateral tail region of a male that expresses an *unc-17::GFP* reporter construct. The scale bar equals 20 μm. The male in the image is in L4 lethargus. The right PCB and PCC postcloacal sensory neurons and SPC spicule motor neuron in addition to some ray neurons express the reporter gene.

L-type voltage-gated calcium channel gene causes 100% spontaneous spicule protraction (n = 200 males). Ablation of PCB, PCC, SPC, and the Anal Dep (n = 36 males) did not abrogate protraction; this observation rules out the possibility that drug insensitivity is due to indirect effects. Thus, since cholinergic secretion is required for aldicarb response and aldicarb-induced protraction requires PCB, PCC, and SPC, we infer that these neurons use ACh to control protractor contraction.

ACh Agonists Act Independently of PCB, PCC, and SPC Neurons

If ACh from PCB, PCC, and SPC neurons acts on the protractors, LEV, NIC, and ARE should induce protraction in the absence of these cells. We found that ablating PCB, PCC, SPC, and the Anal Dep in late L4 males did not abolish drug-induced spicule protraction, even though the ablations reduced the males' ability to sense the vulva and to insert their spicules during mating (10/10 males fail to stay at the vulva for >5 s, 0/10 could insert their spicules within 10 min of observation). At 1 mM LEV, NIC, and ARE, 100% (n = 10 males), 93% (n = 69), and 91% (n = 89) of males, respectively, protracted their spicules. At the same concentration, LEV, NIC, and ARE also caused 95% (n = 20), 85% (n = 20), and 90% (n = 20) of quadruple-ablated males, respectively, to protract their spicules. This result is consistent with ACh activating the protractors and/or additional cells involved in activating protraction.

ACh Agonists Utilize Different Receptors to Stimulate Protraction

To ask if ACh activates protractor contraction through one or more pathways, we assayed agonist-induced protraction in different genetic backgrounds. By comparing the effects of mutations on the EC_{90} of the drugs, we find that ACh utilizes different receptors to stimulate protraction. LEV and NIC activate nicotinic ACh receptors (nAChR) in *C. elegans* (Lewis et al., 1980b). *unc-38* and *unc-29* encode nAChR α and non-α subunits, respectively (Fleming et al., 1997), and mutations in these genes confer LEV resistance (Brenner, 1974; Lewis et al., 1980a). We found that *unc-38(sy576) unc-29(e1072)* double mutants as well as *unc-38* and *unc-29* single mutants did not protract their spicules in LEV (Table 2), indicating that LEV activated protraction through these receptors. We then assayed *unc-38 unc-29* males in other agonists to ask if UNC-38 UNC-29 receptors were the only type of AChR functioning in protraction behavior. The double-mutant males behaved in NIC and ARE similarly to wild-type males (Table 2), demonstrating that these drugs act on other receptors.

ARE is implicated in activating muscarinic ACh receptors (mAChR) that are coupled to Gqα-mediated signal transduction. In *C. elegansI*, *egl-30* encodes Gqα (Brundage et al., 1996). ARE requires Gqα (EGL-30) to induce ACh release from cholinergic motor neurons (Lackner et al., 1999). We tested if ARE utilizes EGL-30 to promote spicule protraction, and we found that wild-type EGL-30 levels were not necessary. However, EGL-30 is required for LEV-induced protraction; the EC_{90} value of LEV for *egl-30(ad805)* males was ~18-fold higher than wild-type (Table 2).

Although LEV-induced protraction required EGL-30, this G protein works redundantly with UNC-38 in promoting NIC- and ARE-induced behavior. *unc-38(sy576)* or *egl-30(ad805)* males behaved in NIC and ARE similarly to wild-type males; however, *egl-30(ad805) unc-38(sy576)* double mutants responded poorly to these drugs. EC_{90} value of NIC and ARE was 5- and 10-fold, respectively, greater than the wild-type value (Table 2). Therefore, at least two signaling pathways must work redundantly to accomplish ARE-induced spicule muscle contraction.

ACh Receptors Signal to UNC-68 and EGL-19 Calcium Channels Differentially

The *unc-68*-encoded ryanodine receptor sarcoplasmic calcium channel and the *egl-19*-encoded L-type voltage-gated calcium channel α1 subunit are utilized by body wall and pharyngeal muscles to facilitate contraction (Maryon et al., 1996, 1998; Lee et al., 1997). To address how these channels are used in protraction, we assayed drug sensitivities of *egl-19* and *unc-68* mutants. Others have reported that *unc-68* mutants are partially resistant to LEV (Lewis et al., 1980a), and we observed that LEV required *unc-68* to induce protraction; the EC_{90} of LEV for *unc-68(r1158)* males was >500-fold higher than for wild-type males (Table 2). However, LEV-induced protraction did not strongly require wild-type EGL-19; the EC_{90} value of LEV for males containing the reduction-of-function mutation *egl-19(n582)* was ~2-fold higher than the wild-type value (Table 2). In contrast to LEV, ARE utilizes EGL-19 more than UNC-68. The EC_{90} value for *unc-68* males was similar (~2-fold higher) to wild-type males, whereas the EC_{90} value *egl-19(n582)* males was >10-fold higher than the wild-type value. ACh recep-

tors stimulated by NIC differ from LEV and ARE receptors in that both channel genes were needed for protraction. The EC_{90} of NIC for *unc-68* and *egl-19(n582)* males was ≥23-fold higher than wild-type males. Thus, ACh receptors involved in spicule protraction mobilize calcium differently to induce muscle contraction.

UNC-38, UNC-68, and EGL-19 Function in Muscles to Facilitate Agonist-Induced Protraction

To test if UNC-38, UNC-68, and EGL-19 in the protractors promote contraction, we restricted expression of *unc-38*, *unc-68*, and *egl-19* to muscle and asked if agonist sensitivities were restored in mutants. The *myo-3* promoter drives transcription of the myosin heavy-chain A gene in nonpharyngeal muscles (Moerman and Fire, 1997). We made two sets of *myo-3* promoter-genomic DNA fusion constructs for each gene: one with and one without GFP fused to the protein's C terminus. EGL-19::GFP expressed from the *myo-3* promoter was visualized in nonpharyngeal muscles of males and hermaphrodites. UNC-38::GFP expressed from the *myo-3* promoter was also visualized in nonpharyngeal muscles, and also in seam cells; the expression in seam cells may be due to transcriptional enhancers within the *unc-38* intron or exon sequences (data not shown). GFP::UNC-68 fused to the *myo-3* promoter has been shown to be expressed in body wall and male sex muscles (Maryon et al. 1998).

Muscle expression of non-GFP-tagged *unc-38*, *unc-68*, and *egl-19* restores LEV, NIC, and ARE sensitivity to *unc-38*, *unc-68*, and *egl-19* mutant males (Table 2), consistent with ACh activating the protractors through these gene products. However, the transgenes do not rescue drug sensitivity of the mutants to wild-type levels. This observation may be due to expression differences between the *myo-3* promoter and *unc-38*, *unc-68*, and *egl-19* endogenous promoters or to an additional neuronal requirement for these genes.

UNC-68 and EGL-19 Control the Two Modes of Spicule-Insertion Behavior

We hypothesized that the drug assays reveal ACh-signaling pathways that facilitate the protractors' distinct contractile behaviors. We tested this possibility by measuring how *unc-68(r1158)* and *egl-19(n582)* mutations affect protractor behaviors. We observed that prodding behavior of *unc-68* males was retarded; the protractors contracted at an average frequency of 0.48 ± 1.4 Hz (n = 4 males; Table 1). All assayed *unc-68* males extended their spicules into the vulva and ejaculated despite their reduced prodding frequency. Expression of wild-type *unc-68* from the *myo-3* promoter restored the contraction frequency to 7.5 ± 1.4 Hz (n = 4 males; Table 1), indicating that UNC-68 regulates contraction frequency in the muscle. We assayed the mating efficiency of *unc-68* males to ask how well they inserted their spicules: despite the severe reduction in prodding frequency, *unc-68* males mated slightly less than wild-type males (*unc-68* mating efficiency [ME] was 0.46 ± 0.18, mean ± SD, n = 5 trials; wild-type ME was 0.85 ± 0.03, n = 6 trials), indicating competence for spicule insertion.

In contrast, *egl-19(n582)* males prodded the vulva at 5.0 ± 1.5 Hz (n = 5 males), similar to but slightly slower than wild-type (p = 0.03; Table 1); however, none of the assayed *egl-19(n582)* males inserted their spicules into the vulva. Also, we did not observe any progeny sired from *egl-19(n582)*males in our mating efficiency tests [*egl-19(n582)* ME = 0.0, n = 7 trials]. However, *egl-19(n582)* males expressing wild-type *egl-19* from the *myo-3* promoter inserted their spicules and ejaculated (n = 7 males) into hermaphrodites. EGL-19 expressed in muscle was also sufficient to restore mating ability to *egl-19(n582)* males [*egl-19(n582);syEx*[*pmyo-3::egl-19*] ME = 0.39 ± 0.18, n = 3 trials]. Thus, wild-type *egl-19* is required more for promoting prolonged rather than periodic protractor contractions.

To ask if a severe loss-of-function mutation in *egl-19* has stronger affects on periodic contractions, we rescued the embryonic lethal *egl-19(st556)* allele (Williams and Waterston, 1994; Lee et al., 1997) with a transgene array containing wild-type *egl-19* DNA and *pmyo-3::gfp* (Figure 5A). We then looked for males with a mosaic loss of the array in the M cell lineage (Figure 5B). The M cell lineage produces some body wall muscle and all male-specific sex muscles (Sulston et al., 1980). We found eight mosaic males out of 2155 males with a loss of the transgene in the M lineage. All mosaic *egl-19(st556) syEx515* males had abnormal tails, with variable defects in the spicules, hook, cloaca, and bursa (data not shown). Six of the males had a functional hook and spicules. During mating, all six males were variably defective in responding to the hermaphrodite, backing along her body, turning, and locating the vulva (unpublished data); however, when the males were able to stop at the vulva, their spicules prodded at a frequency similar to *egl-19(n582)* males (Table 1). Thus, EGL-19 acting in the protractors and retractors is not essential for prodding behavior.

The Anal Dep of one mosaic male was badly reorganized and resembled the Anal Dep of hermaphrodites and larval males (Figure 5D). When wild-type males defecate, the Anal Dep that is attached to the protractors causes the spicules to twitch. The mosaic male with the abnormal Anal Dep had inert spicules during defecation (n = 5 observed consecutive defectation events) suggesting that the Anal Dep was not properly attached to the spicule muscles. Despite abnormal attachment of the Anal Dep to the protractors, the spicules of the mosaic male prodded the vulva at a frequency similar to its other mosaic siblings (Table 1; Figure 5C), suggesting that the EGL-19-containing Anal Dep is not essential to promote periodic contractions of EGL-19-deficient protractors. To address this further, we isolated three additional M cell mosaic males out of 657 males and ablated their Anal Dep. We found that their spicules still prodded the vulva, albeit slightly slower than the nonablated mosaic males (Table 1). We conclude that EGL-19 in the protractors promotes prolonged contraction and has a minimal role in periodic contractions as compared to UNC-68.

Discussion

Execution of Spicule-Insertion Behavior

We have dissected a step in *C. elegans* male mating behavior to learn how an organism executes a genetically encoded task. Based on our observations, we pro-

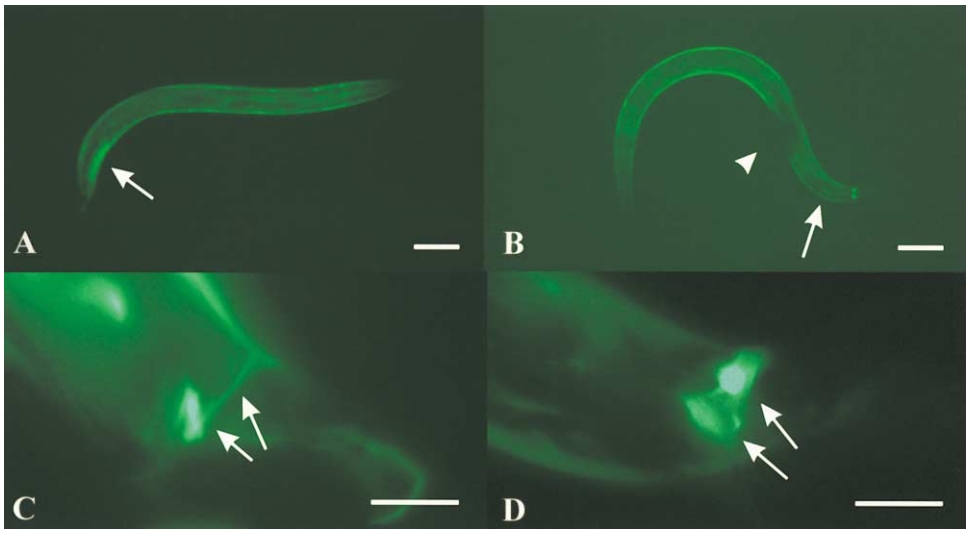

Figure 5. GFP Expression in Muscles of *egl-19(st556); syEx515* Sex Muscle Mosaic Males

(A) Fluorescence image of an *egl-19(st556); syEx515* male that shows transgene-expressed GFP in body wall muscles and sex muscles. The arrow points to GFP expression in the M lineage-derived diagonal muscles. The scale bar equals 0.1 mm.

(B) Fluorescence image of an *egl-19(st556; syEx515* mosaic male that shows loss of transgene-expressed GFP in M lineage-derived body wall muscles (arrow head) and diagonal muscles (arrow). The scale bar equals 0.1 mm.

(C) Fluorescence image of the tail of male #5 that lost *syEx515* in the M lineage (Table 2). The arrows point to the reorganized Anal Dep. The fluorescence of the Anal Dep indicates it has the *egl-19* and *pmyo-3::GFP*-containing *syEx515*. The scale bar equals 20 μm.

(D) Fluorescence image of an incomplete organized *syEx515*-containing Anal Dep in the male that lost *syEx515* in the M lineage (Table 2). The arrows point to the abnormal Anal Dep. The scale bar equals 20 μm.

pose a hypothesis that explains spicule-insertion behavior. When the hook sensillum and the p.c.s. contact the vulva, the spicules prod the vulval slit. Prodding behavior, which results from periodic protractor contractions, continues until the male cloaca shifts off the vulva or the spicules penetrate. The hook and the p.c.s. neurons have been shown to regulate backward locomotion upon recognizing the vulva (Liu and Sternberg, 1995; Barr and Sternberg, 1999). We suggest that, in addition to regulating backing behavior, these two sensilla initiate prodding independently as a consequence of vulva stimulation; this implies that the same cells can control the termination and initiation of sequential steps in a behavior.

We speculate that prodding behavior allows the spicules to pry apart the vulva lips and resets insertion behavior if a spicule thrust fails to penetrate. The behavioral switch from prodding to penetration requires the SPC motor neurons. The neurons innervate the protractor muscles and are required to induce prolonged, but not periodic, muscle contractions. In addition to innervating the protractor muscles, the SPC neurons have sensory-like endings that are attached to the same muscles. These endings have been suggested to have a proprioceptive function (Sulston et al., 1980), and we speculate they mediate the shift from periodic to prolonged muscle contraction. During prodding, SPC could monitor the length of the protractors to detect if the vulva barrier has been breached. Shortening of the protractors due to partial spicule penetration would stretch the attached SPC sensory ending and subsequently trigger the neuron to induce prolonged muscle contraction.

Although the SPC neurons trigger prolonged protractor contraction, sperm and seminal fluid transfer lengthens the duration. After the vulva is penetrated, the protractor muscles remain contracted for ~1 min. Removing the male gonad or triggering the release of sperm from the seminal vesicle but blocking its drainage from the vas deferens to the cloacal opening causes the protractors to relax prematurely. Although the ablations might indirectly affect the development of the spicule muscles, we suggest that a chemical or mechanical property of exiting sperm and seminal fluid lengthens prolonged muscle contraction. This mechanism would insure that males do not withdraw their spicules before ejaculation is completed.

Acetylcholine Activation of Spicule Protraction

We propose that neurons required for spicule-insertion behavior use ACh as part of their repertoire of neurotransmitters to activate the protractors. The *unc-17::GFP* expression pattern and aldicarb pharmacology suggest that ACh transmitted from PCB, PCC, and SPC activate the muscles. The SPC neurons innervate the protractors, suggesting they directly stimulate the muscles; however, postsynaptic partners for PCB and PCC have not yet been reported. ACh released from these sensory neurons might signal the muscles directly or through other cells.

Although ACh secretion from the PCB, PCC, and SPC neurons is important for spicule-insertion behavior, other factors also control the protractors. Additional cholinergic neurons must stimulate protractor contractions, since laser ablation of PCB, PCC, and SPC does not compromise aldicarb-induced protraction as severely as ACh transmission mutations. These unidentified cholinergic neurons could function downstream of the hook sensillum. Also, since PCB and PCC facilitate

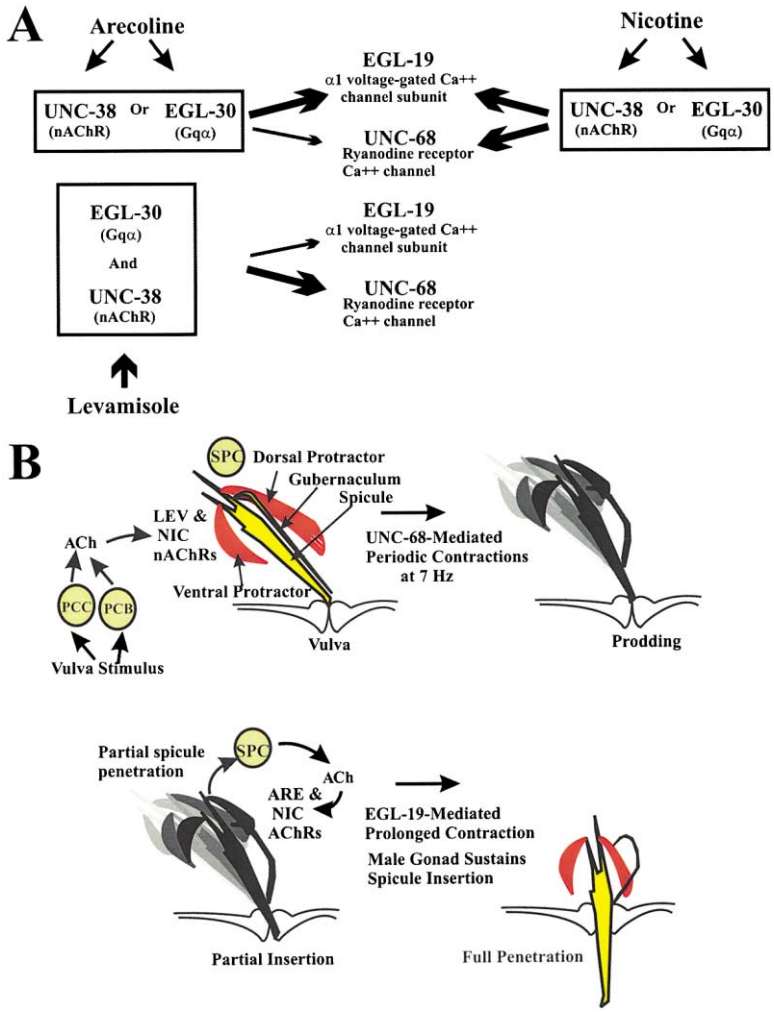

Figure 6. Model of Gene Interactions and Spicule-Insertion Behavior

(A) Summary of genetic interactions involved in drug-induced spicule protraction. The genes within a box are considered a set and are not in any genetic order. The "and" and "or" refer to whether both genes are required ($\geq$5-fold increase in EC_{90} in single mutant backgrounds) or act redundantly ($<$5-fold increase in EC_{90}), respectively, for drug action. Calcium channel genes are postulated to act downstream or parallel to genes in the box. Thick arrows and thin arrows denote major ($\geq$5-fold increase in EC_{90}) or minor ($<$5-fold increase in EC_{90}) gene requirements, respectively, for drug action.

(B) The vulva stimulates male PCB and PCC neurons to secrete ACh. Activated LEV- and NIC-sensitive AChRs on the protractors transmit their signals to UNC-68. UNC-68-mediated calcium mobilization facilitates periodic contractions that cause the spicules to prod the vulval slit. Partial spicule penetration of the vulva triggers male SPC neurons to secrete ACh. ARE- and NIC-sensitive AChRs on the protractors transmit their signals to EGL-19. EGL-19-mediated calcium mobilization promotes prolonged contraction that causes the spicules to penetrate the vulva completely. Sperm transfer sustains prolonged protractor contraction.

periodic contractions whereas SPC induces prolonged contraction, some additional property inherent to these neurons must modulate the muscles. PCB, PCC, and SPC might secrete ACh at different amounts and rates, or they might differentially release cotransmitters that modulate the protractors' response to ACh.

The ACh agonists LEV, NIC, and ARE also trigger the protractor to contract. PCB, PCC, and SPC neurons are not essential for the LEV-, NIC-, and ARE-induced protraction and, thus, the agonists likely activate the protractors and/or other protractor-stimulating neurons.

Multiple Cholinergic Pathways Control Protraction

The *C. elegans* genome encodes ~42 ACh receptor genes, but the behavioral relevance of many of these genes is unknown (Bargmann, 1998). Our genetic analyses of LEV-, NIC-, and ARE-induced protraction suggest that different receptors activate distinct pathways that promote protractor contraction (Figure 6A).

LEV, NIC, and ARE can directly stimulate many *C. elegans* muscles to contract (Brenner, 1974; Lewis et al., 1980b; Trent et al., 1983; Avery and Horvitz, 1990; Avery 1993; Brundage et al., 1996; Miller et al., 1996b). We suggest that AChRs defined by these drugs also function in the protractors. LEV binds to UNC-38 UNC-29 nAChRs in *C. elegans* (Fleming et al., 1997), and additional muscle nAChRs activated by NIC have been described in detail using electrophysiological methods (Richmond and Jorgensen, 1999). ARE-sensitive receptors have been implicated in stimulating pharyngeal pumping (Avery, 1993). Mutations that increase Gqα(EGL-30) activity sensitize the pharynx to ARE, suggesting that the drug activates G protein receptors (Brundage et al. 1996; Robatzek et al. 2001). ARE receptors have not been identified in *C. elegans*, but the drug activates both nAChRs and mAChRs in insect neurons (Tribut et al., 1994), demonstrating that ARE's mode of action is nonspecific.

UNC-38 and Gqα(EGL-30) are required differentially for the action of all three drugs tested in this report. LEV- and ARE-induced protraction utilize UNC-38-containing nAChRs, but ARE likely activates additional nAChRs. LEV stimulation also requires Gqα(EGL-30), but since LEV probably does not directly activate Gqα(EGL-30)-coupled mAChRs, Gqα(EGL-30) might act independently of the agonist either upstream or parallel to drug activation by sensitizing the muscle to stimulation. In contrast, at the EC_{90} concentration, ARE- and NIC-induced protraction require either UNC-38 nAChRs or Gqα(EGL-30)-coupled receptors. ARE might directly activate Gqα(EGL-30)-

coupled mAChRs and nAChRs, and both muscarinic and nicotinic pathways redundantly induce protraction. Alternatively, ARE and NIC might only activate nAChRs, and Gqα(EGL-30) could potentiate drug-induced protraction indirectly by sensitizing the muscle to the agonists. In this scenario, ARE and NIC do not require Gqα(EGL-30) as much as does LEV, since they might activate multiple nAChRs, and the additive activation of many receptors might tolerate a mutation-induced drop in Gqα(EGL-30) levels.

Muscle AChRs transduce the agonist signals differentially to downstream calcium channels. UNC-68 sarcoplasmic calcium channels facilitate LEV-induced protraction, whereas EGL-19 voltage-gated calcium channels promote the ARE response. In contrast, both channels are needed for NIC-induced protraction. During mating, UNC-68 and EGL-19 differentially promote periodic and prolonged protraction contractions. These observations imply that different AChRs regulate the protractors' distinct contractile behaviors through these channels (Figure 6B). During periodic contractions, ACh transmitted from the PCB and PCC neurons might activate UNC-38 UNC-29 nAChRs and NIC-activated nAChRs that then cause UNC-68 to release intracellular calcium. During prolonged protractor contraction, secretion of ACh from the SPC neurons might activate ARE-sensitive receptors, which, in parallel with UNC-38/UNC-29 and NIC-activated nAChRs, trigger influx of extracellular calcium through EGL-19 voltage-gated channels. The amount or duration of calcium signaling through EGL-19 likely causes the protractors to shorten completely.

Experimental Procedures

General Methods and Strains

C. elegans strains were cultured at 20°C using standard protocols (Brenner, 1974). All strains used in this study contain *him-5(e1490)* on LGV (Hodgkin et al., 1979). The wild-type reference strain PS1395 contains *plg-1(e2001)* on LGIII (Hodgkin and Doniach, 1997). Additional alleles used were *egl-30(ad805)*, *unc-29(e1072)* for LGI; *unc-52(e444)* for LGII; *pha-1(e2123ts)*, *unc-64(e246)* for LGIII; *egl-19(n582)*, *egl-19(n2368)*, *egl-19(st556)*, *unc-31(e169)* for LGIV; and *unc-68(r1158)* for LGV (Riddle et al., 1997). *unc-38(sy576)* on LGI was generated by standard ethylmethanesulfonate mutagenesis (Brenner, 1974). Virgin males were used for all experiments. Mid-L4 males were isolated from hermaphrodites and were kept in groups of 20–50 animals per culture plate prior to observation.

Laser ablations were conducted using a standard protocol (Bargmann and Avery, 1995). To ablate the hook sensillum, the hook progenitor cells P9.p and P10.p were killed at early L3 stage. PCA, PCB, PCC, SPC, and Anal Dep ablations were performed either on early L4 males at a developmental stage prior to when the tail ventral hypodermis shrinks and migrates anteriorly or on late L4 males after the tail ventral hypodermis completely retracts and the spicules start to form. The effects of ablations on quantitative drug response differ if the operations were conducted at early versus late L4. Early ablations reduce drug sensitivities ~30% relative to late ablations (data not shown). We speculate that ablations of presynaptic cells too early in development limit the amount of drug-responsive receptors on their postsynaptic partners. To cauterize the spicule tips, young virgin adult males were anesthetized in 5 mM sodium azide, which caused spicules to protract. The laser was aimed so that protruding regions of the spicules were cut off. Gonad ablations were performed on early L1 animals. Linker cell ablations were conducted on early L4 animals when the migrating linker cell was arbitrarily close to the proctodeum. Other ablations conducted were VD12 n = 8 males; VA12 n = 9; AS11 n = 2; DD6 n = 2; DA9 n = 2; DA8 n = 2; PVX n = 4; VD13 n = 4; PDA, DA9 n = 10; AS10, 11 n = 9; VA10, 11, 12 n = 4; VD11, 12, 13 n = 4; VD13, PVX n = 3; VA 8, 10, 11, 12, DA6 n = 1; DA8, 9 n = 6, CA5, 6, 7, 8, 9, CP5, 6, 7, 8, 9 n = 6; DA 5, 6, 7, 8, 9, DD4, 5, 6, DB6, 7 n = 4; PCC, SPC, Anal Dep, PDC, DX3, VD11, 12, DD6, VD13, VA11, 12, DA8, 9, DB7 n = 2; PCC, SPC, Anal Dep, PGA, PVZ, EF1, 2, 3, 4, DX1, 2, 3 n = 2; PGA, PVZ n = 4; SPV, SPD, SPC, CA5, 6, 7, 8, 9, CP5, 6, 7, 8, 9 n = 1; and SPV, SPD, SPC, Anal Dep, AVL, DVB, n = 3. Operated males were kept in groups of 5–10 males, and their behaviors were observed 18–24 hr after L4 lethargus.

Observations of Spicule-Insertion Behavior

Mating behavior was viewed through a 40× objective using a compound microscope mounted with an MTI CCD72 black and white video camera and recorded with a Panasonic AG-6740 time-lapse Super-VHS videocassette recorder. Mating lawns (5 mm diameter) used in observations were made by spotting 1 μl of *Escherichia coli* OP50 culture onto a NG agar plate. Five hermaphrodites and one male were placed on the lawn, and a 1 cm block of agar containing the mating lawn was placed on a microscope slide for direct viewing. A fresh mating lawn was used for every observation. *unc-38(sy576); egl-19(n582)* hermaphrodite adults (24 hr post L4 lethargus) were used in all matings. The camera was focused on the vulva, and the recorder was activated when the male tail touched the vulva. To quantify the rate of spicule twitches for wild-type, SPC-ablated, and *egl-19* males, recordings were played back at 1/7 original speed, and the downstrokes the spicules made in 1 s were counted manually. Since the male tail occasionally shifted in and out of camera focus, not all sequential twitches could be counted, but only runs of focused spicule movements were quantified. For matings using *unc-68* males, recordings were played back at original speed and the time between spicule downstrokes was measured manually. The Wilcoxon (Mann-Whitney) test was used to determine statistical significance.

Measuring the Duration of Prodding Behavior

Late L4 hermaphrodites were picked to fresh plates. Every 24 hr thereafter, ten hermaphrodites were tested for vulva penetration. The hermaphrodites were placed with an 18 to 24 hr virgin adult male onto a mating lawn, and observed with a Wild M5A microscope. After initiation of mating, a stopwatch was started when the male tail touched the vulva. If the male tail shifted off the vulva, the timing was temporarily stopped. Timing was resumed when the male tail repositioned itself over the vulva. The observation was terminated and recorded when the spicules completely inserted or 5 or 10 minutes of prodding transpired.

Measuring the Duration of Spicule Insertion

Five *unc-31* hermaphrodite adults (24–48 hr post L4 lethargus) were placed with a male onto a mating lawn and observed with a Wild M420 microscope. Timing was started when the male inserted his spicules into a hermaphrodite and was stopped when the base of the spicule began to retract back into the male. Spicule insertion events where the male detaches prematurely from the hermaphrodite prior to spicule retraction were ignored. The Wilcoxon (Mann-Whitney) test was used to determine statistical significance.

Pharmacology

LEV, NIC, and ARE were purchased from Sigma and aldicarb from Chem Services, West Chester, PA. LEV, NIC, ARE, and aldicarb were prepared in distilled water and kept frozen at –20°C as 100 mM, 6 mM, 100 mM, and 5 mM stock solutions, respectively. Aliquots of a drug were thawed and serially diluted in distilled water as needed, and 1 ml of the drug was placed in a Pyrex, round-bottom, 3 well titer plate. Five to ten males were transferred to the bath, and the males were observed for 5 min with a Wild M5A microscope. Males were scored as responsive to the drug if their spicules stayed protracted for ≥10 s. Fresh drug baths were used after every three trials. EC_{90} values were estimated by converting the percentages of males that protracted to probit values (Finney, 1971). Probits were then plotted against the log of the drug concentration and the curve was fitted by linear regression using the least squares method (data not shown). The estimated EC_{90} corresponded to the fitted probit of 6.28. To test for aldicarb responsiveness, ablations were con-

ducted at mid- to late-L4 stage and adults were then tested. Five to ten males were added to 1 ml of 5 mM aldicarb and were observed for 15 min; the times were recorded when their spicules protracted.

Mating Efficiency Tests

The mating efficiency test was adapted from Hodgkin (1983). Six males and six *unc-52* paralyzed hermaphrodites were put on a standard OP50-seeded NG plate, and each plate was considered a mating trial. The total number of paralyzed and nonparalyzed cross progeny was counted 3-4 days later. Mating efficiencies were calculated as the percentage of cross progeny over total progeny.

Gene Constructions

A 7832 bp *PstI-BamHI unc-17*-containing fragment of cosmid ZC416 (Alfonso et al., 1993; Coulson et al., 1986) was inserted into the *PstI-BamHI* sites in pPD95.75 (all vector plasmids courtesy of A. Fire, Carnegie Institute of Washington) to make pR14. The GFP sequence was fused in frame to the last exon of *unc-17*. Upstream sequences of *unc-17* were added by inserting a 8755 kb *PstI* fragment of ZC416 into the *PstI* site of pR14 to make pR18. pR18 (100 ng/μl) and the wild-type *pha-1* 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). A total of 20 mid-L4 males from two transgenic lines were analyzed for *unc-17* expression.

egl-19 and *egl-19::GFP* fused to the *myo-3* promoter were made as follows. *egl-19* DNA was PCR amplified from N2 using primers 5′-GCGTTAACCTGTGTCCATCATGTCAGG-3′ and 5′-GGAAGATGAT GGTTCTCAAAGAGTTGTAACTAAAAGTAGATC-3′ or 5′-AAGAGTTGT AACTAAAAGTAGATCTTCTTGTGATCCTCCGCG-3′. Genomic *egl-19* containing a stop codon was blunt-end ligated to *Kpn*I-cut, filled-in pPD96.52 (p*myo-3* vector) at a molar ratio of 34:1, PCR product to *myo-3* promoter vector. To make a C-terminal fusion of GFP to *egl-19* expressed from p*myo-3*, genomic *egl-19* lacking a stop codon was three-way ligated to *Kpn*I-cut, end-filled pPD96.52 and to *Sma*I-cut pPD95.75 at a molar ratio of 72:1:0.5, PCR product to *myo-3* promoter vector to GFP vector. The ligation mixtures were precipitated, dissolved in water, and injected into *egl-19(n582)*; *him-5*. p*myo-3::egl-19* ligation mix was injected at 300 ng/μl with the p*myo-2:GFP* plasmid pPD118.33 (10 ng/μl). p*myo-3::egl-19::GFP* ligation mix was injected at 600 ng/μl. Two lines were obtained for both sets of injections; *syEx476* (p*myo-3*::*egl-19::GFP*) and *syEx465* (p*myo-3*::*egl-19*) were analyzed.

unc-38 and *unc-38::GFP* fused to the *myo-3* promoter were made as follows. *unc-38* DNA was PCR amplified from N2 using the primers 5′-ATGCGCTCTTTTTGGTTATTCCTTTTACTGTTATTATTTTGC ATCTC-3′ and 5′-TCAGAAACTAATTGGATTAGCAGATAAATTGGCT GGACGATACT-3′ or 5′-GAAACTAATTGGATTAGCAGATAAATTGGC TGGACGATACTGC-3′. *unc-38* DNA with a stop codon was blunt-end ligated into *Nhe*I-cut, end-filled pPD96.52 to make pR29. To make GFP fused to the C terminus of *unc-38*, *unc-38* DNA lacking a stop codon was blunt-end ligated to *Sma*I-cut pPD95.75 to create pR26. pR29 was injected (50 ng/μl) into *unc-38; him-5* with pPD118.33 (10 ng/μl). To make worms expressing *unc-38::GFP* from the *myo-3* promoter, pR29 (50 ng/μl) and pR26 (50 ng/μl) were coinjected into *unc-38; him-5*. p*myo-3::unc-38::GFP* was generated through homologous recombination between the plasmids. Two transmitting lines were obtained from each injection. *syEx469* (p*myo-3:unc-38*) and *syEx470* (p*myo-3:unc-38::GFP*) were analyzed for this report.

unc-68; him-5 containing *syEx475* (p*myo-3::unc-68*) was made following the injection procedure in Maryon et al. (1998).

syEx515 Construction and Mosaic Analysis

egl-19 promoter and coding region was PCR amplified from N2 DNA using upstream primer 5′-CGGGATCCATACTTCTTCCATTTCCTAA CCCGC-3′ and downstream primer 5′-GGAAGATGATGGTTCTCAAA GAGTTGTAACTAAAAGTAGATC-3′. The PCR product was injected (30 ng/μl) into *egl-19(n582); him-5* with pPD93.97 (10 ng/μl). A fluorescing rescued line containing *syEx515* was crossed into *egl-19(st556)/ unc-82(st1323) unc-24(e138)* to eventually obtain *egl-19(st556); him5; syEx515*. L4 males with fluorescence missing in segments of their body wall muscles were identified under UV light using a dissecting microscope and were separated from hermaphrodites. In some of the males, the Anal Dep muscle was ablated. Adults were later scored for loss of fluorescence in the sex muscles. Mosaics were then assayed for mating behavior and then analyzed further under UV light using a compound microscope.

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