A CHOLINERGIC SENSORY-MOTOR CIRCUIT

CONTROLS THE MALE COPULATION BEHAVIOR IN *C. ELEGANS*

A Dissertation

by

YISHI LIU

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2011

Major Subject: Biology

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Approved by:

Chair of Committee,	L. Rene Garcia
Committee Members,	James Grau
	Brian Perkins
	Michael Smotherman
Head of Department,	Jack McMahan

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ABSTRACT

A Cholinergic Sensory-motor Circuit Controls the Male Copulation Behavior

In *C. elegans*. (May 2011)

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Chair of Advisory Committee: Dr. L. Rene Garcia

The nervous system coordinates a sequence of muscle movements to give rise to animal behaviors. In complex invertebrates or lab-studied vertebrates, due to the large number of cells in their nervous systems and the complexities of their behaviors, it is difficult to address how circuits process information to direct each motor output of the behavior. In this dissertation, I used the *Caenorhabditis elegans* male copulation behavior as a model to address how a compact circuit coordinates different behavioral programs.

Insertion of a male copulatory organ into a suitable mate is a conserved behavioral step for most terrestrial mating. However, the detailed molecular and cellular mechanisms for this distinct social interaction have not been elucidated in any animal. During mating, the *C. elegans* male cloaca is positioned over the hermaphrodite's vulva as he attempts to insert his copulatory spicules repetitively. Rhythmic spicule thrusts cease when insertion is sensed. Circuit components consisting of sensory/motor neurons and sex muscles for these steps have been previously identified, but it was unclear how their outputs are integrated to generate a coordinated behavior.

Here, I delineate cellular and molecular mechanisms that transduce sensory information into coordinated behavioral programs during male mating. I show that contraction of the male oblique muscles is required to sustain genital contact between the sexes. These muscles are innervated by the cholinergic postcloacal sensilla (p.c.s.) sensory/motor neurons. The ionotropic AChRs that contain either the ACR-16 or the UNC-29 subunit mediate synaptic transmission at these neuromuscular junctions; and a $G\alpha_q$–coupled muscarinic AChR, GAR-3, is likely to function presynaptically to enhance the ionotropic AChRs-mediated synaptic transmission. For spicules to rhythmically thrust during genital contact, activity of the oblique muscles and the gubernacular muscles is transmitted to the spicule protractor muscles instantaneously via gap junctions and causes shallow protractor contractions. The rhythmic protractor contractions eventually switch to sustained contraction, as the SPC sensory-motor neurons integrate information of spicule position at the vulva with inputs from the hook and cloacal sensilla. The ERG-like K^+ channel, UNC-103 is likely to set a threshold requirement for integration of these inputs, so that sustained spicule protraction is not stimulated by fewer inputs.

DEDICATION

This work is dedicated to my grandma, Cuiyun Chen and grandpa, Yutang Liu, who set great examples for me. They have been lifetime scholars who devoted their lives to research in agriculture and chemical engineering, respectively.

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I want to express my deepest gratitude to my advisor, mentor and role model scientist, Dr. Luis Rene Garcia. As a tireless teacher and a great mentor, he has been teaching me the right way to do science. He has showed me how to be a good scientist: positive ways of thinking, confidence and a willingness to take risks. I truly appreciate the challenges he's given me throughout these years. Although some of them were hard to take at the beginning, they all proved to be precious moments where I grew the most. From him I learned what optimism is: turning crisis situations into opportunities to excel. As a constant supporter of new ideas, he has been willing to supply what is necessary to carry out experiments. He provided plenty of space and opportunity for me to learn from my experience and mistakes. I also would like to express my sincere respect to him for being who he is; he never demanded more from us just because he was under great pressure, instead he kept pressure on himself and worked harder. When he got a new grant, he celebrated with us and raised our salaries.

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NOMENCLATURE

ACh	acetylcholine
p.c.s.	postcloacal sensilla
AChR	acetylcholine receptor
nAChR	nicotinic acetylcholine receptor
mAChR	muscarinic acetylcholine receptor
EC_{50}	effective concentration to cause response in 50% of the population
EC_{90}	effective concentration to cause response in 90% of the population
LEV	levamisole
oxo M	oxotremorine M
lf	loss of function mutant
o	mutant that has no functional protein of the gene
male sex muscles	male-specific muscles that are likely to regulate male mating
L-AChR	levamisole-sensitive ionotropic AChR
prc	protraction constitutive

TABLE OF CONTENTS

CHAPTER Page

CHAPTER Page

LIST OF FIGURES

Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

How can an organism behave in order to respond properly to its environment and internal needs using a repertoire of genes? The capability of the nervous system to generate a specific behavior largely depends on the **structure** of circuits and the **connectivity** of each pair of excitable cells. Gene products define the circuit structure and eventually function in the context of such a network to give rise to the behavioral output.

My interest, as part of my graduate studies, is to address the following question: How does the nervous system recruit various gene products and diverse functional connections to generate and modulate innate animal behaviors? Specifically, I wanted to study a behavior that is likely to be regulated by a simple circuit, where the function of a single cell can be assigned and the genes expressed on each cell can be studied; while at the same time, the animal's behavior is complex enough that sensory information needs to be integrated and motor outputs need to be coordinated by the circuit. I can then place these gene functions into a context of specific circuit structure and understand how the behavioral pattern is generated by a combination of molecules and functional connections.

This dissertation follows the style of Journal of Neuroscience.

C. elegans as a model organism to study neural basis of behavior

My study uses *C. elegans* as a model organism. This nematode has two sexes, hermaphrodites and males. The hermaphrodite nervous system contains 302 neurons; the male has 383 neurons (Sulston et al., 1980; White et al., 1986). This compact nervous system encodes a variety of general behaviors, such as feeding, locomotion, defecation and sensory modalities in both sexes, as well as sex-specific behaviors, such as the hermaphrodite egg-laying and the male mating behavior (Bargmann, 2006; Barr and Garcia, 2006; Branicky and Hekimi, 2006; Goodman, 2006; Rankin, 2006; Schafer, 2006; Mori et al., 2007; Zhang et al., 2008b; Sengupta and Samuel, 2009; Sokolowski). The circuits controlling these behaviors are comprised of a few neurons and muscles, which can be multifunctional in sensing, processing and producing signals.

Various genetic and surgical manipulations can be done readily in *C. elegans* to study their behaviors. Genetic mutants with specific defective phenotypes can be easily obtained through mutagenesis as a way to study genes that regulate the traits. The nervous system has a predictable cell lineage, so that individual neurons can be recognized and laser-ablated to assess their functions in regulating behavior. Optogenetic technology tools have become available recently to stimulate or suppress excitable cells using high-energy light at any time (Nagel et al., 2005). Genetically encoded Ca^{2+} sensors are used to monitor cell activities in behaving animals (Nakai et al., 2001). Lastly, the wiring of the hermaphrodite nervous system and the male-specific circuits has been reconstructed to reveal physical connections between excitable cells (White et

al., 1986)(S.W. Emmons, personal communication, 2010). With these tools and information, it is possible to address how gene products shape cell physiology, determine circuit function, and eventually give rise to behavior, within a single model organism.

Neural basis for mating behavior in various organisms

The behavior I study is the *C. elegans* male mating behavior. As a motivated stereotyped behavior, male mating is under regulation of a hard-wired behavioral circuit and is also subjected to modulatory mechanisms that fine tune the behavioral output according to external environment and internal state of the animal.

The neural basis of mating behavior has been studied in a variety of organisms, including rodents, birds, leeches, fruit flies and nematodes, where the studies are mostly focused on male mating (except for leeches, which are simultaneous hermaphrodites), since the behavioral responses of females/hermaphrodites are not as obvious (Ball and Balthazart, 2004; Barr and Garcia, 2006; Hull et al., 2006; Villella and Hall, 2008; Wagenaar et al., 2010).

Vertebrate studies heavily focus on the neuroendocrine mechanisms that regulate the male sexual behavior, partly because identification of the sites of action of sex steroid hormones in the brain provides an easier path to identify neural circuits that regulate the mating behavior in the highly complex central nervous system (Balthazart and Ball, 2007; Hull and Dominguez, 2007). Using conventional methods, such as

lesions, measures of cellular activity through detecting immediate early gene expression and electrophysiological stimulation and recordings, distinct brain regions that control different aspects of male sexual behavior have been identified (Ball and Balthazart, 2004; Hull et al., 2006; Balthazart and Ball, 2007; Hull and Dominguez, 2007). In rats, it is demonstrated that the chemical cues sensed by the main olfactory system and the vomeronasal system are the most important sensations for males to initiate mating, though other sensory inputs, such as touch sensation and visual cues, also play a role. The chemosensory information is processed in the medial amygdala, and, coupled with the somatosensory information from the genitals, is sent to the medial preoptic area, where signals from all sensory systems get integrated and then facilitate the sexual behavior by releasing neurotransmitters, such as dopamine. The gonadal hormones are considered as influencing sexual behavior via regulating neurotransmitter release and recycle in these chemosensory pathways (Hull and Dominguez, 2006, 2007). Similar progress has been made in birds, where the basolateral amygdala is considered to regulate the appetitive phase of sexual behavior, also referred as courtship; whereas, the pre-optic area has been identified as a main area for controlling the final phase of sexual behavior, which is copulation (Ball and Balthazart, 2004; Balthazart and Ball, 2007; Fusani, 2008). Nonetheless, identification of neural pathways that receive sensory information and process it into motor output using conventional methods is not easy in vertebrates and is limited in resolution, even though progress has been made through the use of novel tracing techniques (for example, transneuronal tracers expressed in certain neuron population travel to neurons that are postsynaptic and/or postsynaptic to these

neurons; GFP-expressing pseudorabies virus injected into a certain region of brain infect the neurons that are presynaptic to this region)(Boehm et al., 2005; Yoon et al., 2005).

Many intriguing aspects of the vertebrate sexual behavior have been studied based on knowledge of how the nervous system regulates the behavior, such as effects of aging, sexual preference and previous sexual experience (Hull et al., 2006; Mak et al., 2007; Sakuma, 2008; Wu et al., 2009). After the motivation phases (courtship and copulation), the male sexual behavior is inhibited as a result of copulation. This satiation aspect of the behavior has also been studied, to identify neurotransmitters, hormones, and brain areas involved in inhibition of mating after satiety (Lorrain et al., 1999; Phillips-Farfan and Fernandez-Guasti, 2009).These aspects make sexual behavior neurobiology a particularly interesting field, but it also demands more insight into how the behavior is generated and modulated by the nervous system.

Due to the extreme complexity of vertebrate brains, it remains a daunting task to dissect the molecular components of all the circuits that regulate sexual behavior in rodents or birds. This complexity also limits studies that are interested in the dynamics and plasticity of the circuits. In the near future, insight into how genes and signaling pathways, which are recruited by defined nervous system structures, give rise to a coordinated behavior mostly likely will come from studies on genetically tractable organisms with simpler nervous systems, such as *Drosophila melanogaster* and *Caenorhabditis elegans*.

In *D. melanogaster*, studies of mating behavior have primarily focused on the motivated phase, which is courtship. The male courtship starts when a male orients

towards and follows the female. He taps her with his forelegs, vibrates one of his wings to produce a courtship song, and licks her genitalia. Finally, he copulates with her by curling his abdomen and thrusting his genitalia towards her. This sequence of motor outputs prior to copulation is mostly a fixed pattern (Sturtevant, 1915; Crossley and Zuill, 1970). Just like in vertebrates, sensory modalities required for the males to initiate courtship have been studied most intensively (Hall, 1994; Yamamoto and Nakano, 1999). This is probably because mutations that affect specific sensations are more readily found and characterized from a mutagenesis screen of mating defects. In contrast, less is known about how sensory information is relayed and processed in deeper layers of the nervous system and eventually translated into motor outputs. Two major approaches have been employed to map functional brain structures involved in regulating the courtship behavior: 1) analyzing sex mosaics, in which the sexual identity of a specific population of neurons is switched to the opposite sex by expressing sex-specific forms of the *transformer* (*tra*) gene, for example, by turning specific subsets of male neurons into female identity using the *GAL-4* enhancer-trap method (Brand and Perrimon, 1993; Kaiser, 1993), the anterior suboesophageal ganglion for the first time was indicated to control male copulation (Ferveur and Greenspan, 1998); and 2) molecular genetic disruption of neuronal functions, which involves expressing RNAi of a certain master regulatory gene, or expressing dominant alleles of genes that can suppress neural activity in a subset of cells in the brain (Broughton et al., 2004; Manoli and Baker, 2004). These are powerful tools that have revealed the roles of different regions of the *Drosophila* brain in regulating courtship. However, these tools are both

limited by the fidelity and resolution of the enhancer-trap expression patterns (Villella and Hall, 2008).

The study of a single gene, *fruitless* (*fru*), has provided great insight into how *Drosophila* courtship is regulated by the nervous system. This gene encodes a transcription factor, which is demonstrated to be necessary and sufficient for a fly brain (male or female) to generate male mating behavior (Manoli et al., 2005). The fact that *fru* is essential for execution of all steps in courtship (from initial encounter to final sperm transferring), indicates that the cells where it is expressed must play a certain role in these behavioral steps (Manoli et al., 2006). Consistent with this idea, *fru* is expressed in peripheral sensory systems (olfactory, visual, gustatory, auditory, and tactile), all of which have been shown to be involved in initiating the courtship behavior. It is also expressed in about 2000 neurons in the fly's central nervous system (CNS). These neurons form clusters throughout the brain, including areas that have been implicated in courtship regulation. Therefore, the *fru*-expressing cells are likely to regulate different aspects of the courtship (Manoli et al., 2005; Stockinger et al., 2005). Though it seems that most of the male neurons that express *fruitless* have counterparts in the females, and these neurons are wired in a similar way in both sexes, a study has been carried out to find subsets of these neurons that are sex-specific or dimorphic, as an approach to determine the roles of subsets of *fru*-expressing neurons in generating courtship patterns (Kimura et al., 2005; Stockinger et al., 2005; Yu and Dickson, 2006). Nonetheless, specific functions are yet to be assigned to different *fru*-expressing neurons as a way to map the circuitry of courtship in a better resolution.

Using *fru*-based genetic tools and other neurogenetic approaches, a couple of regions of the CNS have been found to control courtship; for example, the antennal lobes facilitate partner recognition; a cluster of cells in the dorsal lateral brain promotes the initiation of courtship; the dorsal posterior brain is involved in multiple steps, including initiation, following, tapping and wing extension; and the posterior midbrain is important to generating licking and copulation (Billeter et al., 2006; Villella and Hall, 2008). However, it still remains unknown how information is processed in these regions so that the original sensory cues can be interpreted into motor outputs that are coordinated sequentially or simultaneously.

C. elegans male mating behavior

With striking similarity to the stereotyped copulation in rodents and fruit flies, the *C. elegans* male mating is a complex multi-step behavior (Fig. 1, adapted from Liu and Sternberg, 1995). A male starts mating when his tail contacts any part of the hermaphrodite cuticle. As major sensory organs required for initiation of mating are located in the male tail, he uses his tail to scan the hermaphrodite cuticle searching for the vulva. During searching, his tail is closely sealed to the hermaphrodite cuticle with the ventral side facing the hermaphrodite, as he moves backwardly. Once he locates his cloaca at the hermaphrodite vulva, he maintains his tail position at the vulva, and thrusts his bilateral pair of copulatory organ, spicules, rapidly (7-11 Hz) towards the vulval slit. A male who performs better in maintaining prolonged and precise contact between his

cloaca and his mate's vulva is more likely to insert his spicules into the vulval slits. If a male loses contact with the vulva prior to spicule insertion, he resumes backward locomotion to locate the vulva again. About 31 seconds after successful spicule insertion, the male ejaculates sperm into the hermaphrodite uterus. The spicules get retracted after sperm transfer (Liu and Sternberg, 1995; Garcia et al., 2001; Schindelman et al., 2006). The *C. elegans* hermaphrodites produce both ovum and sperm, so they do not need to mate in order to reproduce. Probably as a result, they do not participate in mating and even attempt to escape from the males.

The *C. elegans* male has 89 sex-specific neurons and 41 male-specific muscle cells (called "**male sex muscles**") located in the male tail. These cells are presumably used to facilitate different aspects of the male mating behavior (Sulston et al., 1980; Portman, 2007). Previous studies have revealed the roles of a couple of male-specific muscles and neurons in regulating different substeps of the behavior.

The initial contact with hermaphrodites, referred as "response behavior", is mediated by nine pairs of finger-like sensory structures located in the male tail, called **rays** (Fig. 2*A*,*B*). Each ray contains a structural cell (Rnst) and dendrites from two neurons, RnA and RnB. These neurons sense the hermaphrodite cuticle and promote backward locomotion of the male, so his tail can scan along the hermaphrodite, searching for the vulva (Fig. 1*A*)(Liu and Sternberg, 1995; Barr and Sternberg, 1999; Barr and Garcia, 2006). The ray RnB neurons (except for ray 6) are ciliated and are

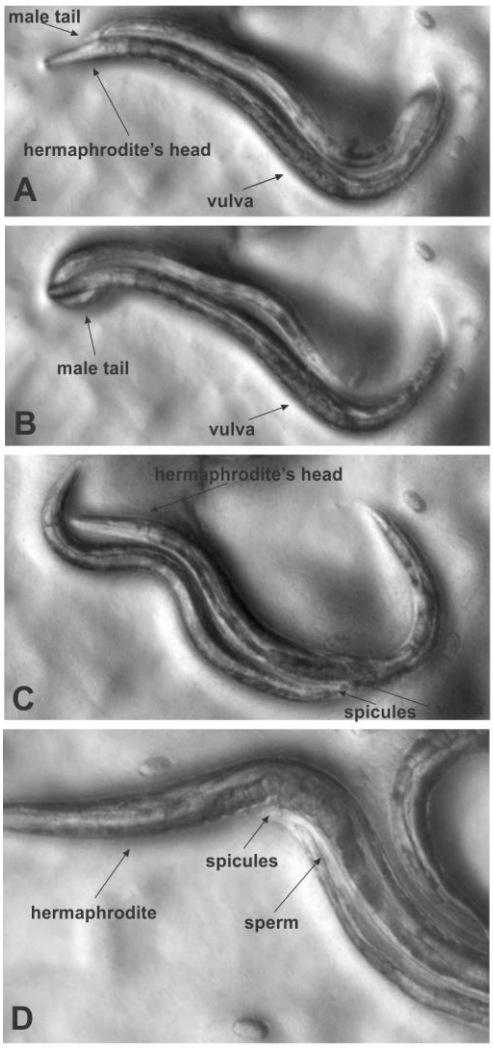

Figure 1. Steps of *C. elegans* male mating.
A. The male (the upper one) moves backward and uses his tail to scan the dorsal side of the hermaphrodite (the lower one) cuticle, searching for the vulva.
B. Once the male reaches the end of the hermaphrodite, he turns his tail to the ventral side of the cuticle and continues scanning.
C. Once his tail senses the vulva, he stops the backward locomotion and places his tail over the vulva. At the mean time, his copulatory spicules rapidly prod at the vulva slit, until they get inserted.
D. A few seconds after spicule insertion, sperm gets transferred.

considered sensory neurons, while the RnA neurons are not ciliated and their tips are held inside of the ray cuticles (Sulston et al., 1980). These neurons form both chemical and electrical connections onto each other, and also send extensive connections to the other components of the male-specific circuit (Male Wiring Project). However, it is unknown what kind of cues are sensed by them and how these sensory inputs get processed by the circuit to regulate mating.

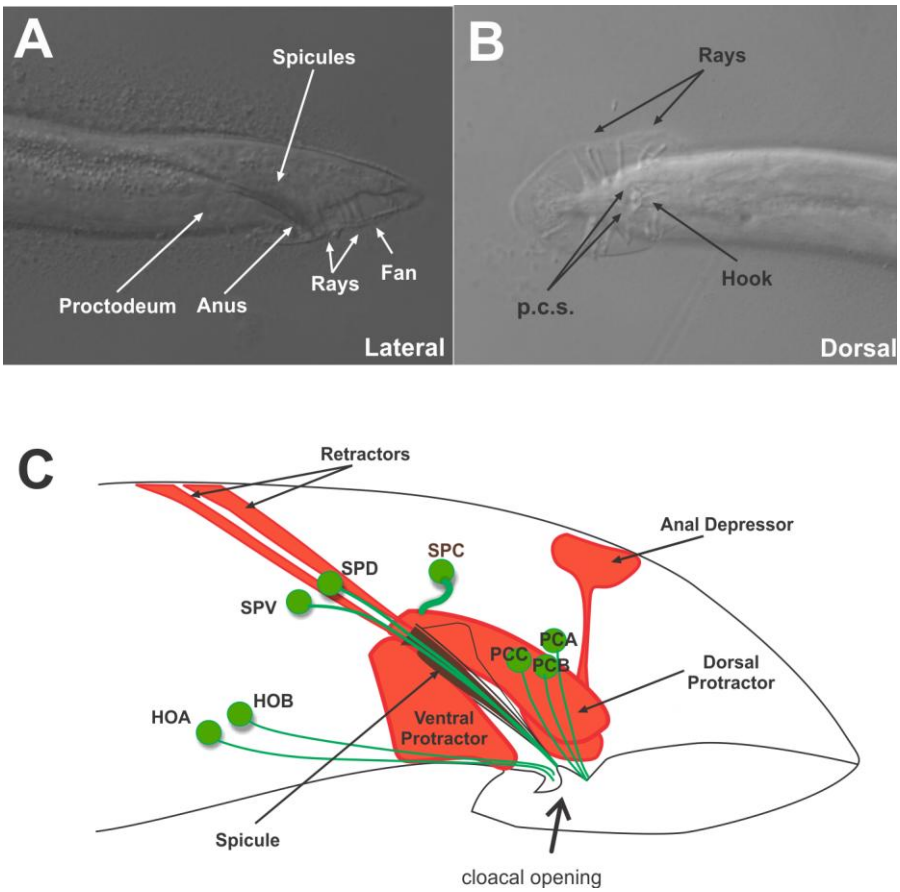

Figure 2. Male tail morphology and anatomy.
A. DIC image of the male tail (lateral view). Distinct structures are labeled.
B. DIC image of the male tail (dorsal view). Distinct structures are labeled.
C. Cutaway representation of neurons and muscles involved in spicule movements.

During vulva searching, upon reaching an end of the hermaphrodite (at the head or the tail), the male curls his tail ventrally to make a sharp turn, in order to approach the other side of her (Fig. 1*B*). This substep is referred as "turning behavior". A subset of rays and the male-specific ventral cord neurons (CP1-CP6) are thought to facilitate this behavior, as males that had disrupted function in these cells either as a result of laser-ablation or genetic mutations displayed defective turning behavior (Loer and Kenyon, 1993; Liu and Sternberg, 1995). The CP neurons have synapses to the male-specific diagonal muscles located at the male tail, and it is suggested that these neurons promote tight ventral curl of the male tail via activating these muscles (Loer and Kenyon, 1993).

The male locates the vulva via mechanosensation (characteristic surface) and chemosensation (secreted chemical cues)(Barr and Garcia, 2006). These are mediated by two types of sensilla, the **hook sensillum** and the **postcloacal sensilla (p.c.s.)**, located anterior and posterior to the cloacal opening, respectively (Fig. 2*B*). The hook sensillum contains two neurons (HOA and HOB) and they probably sense both chemosensory and mechanosensory cues (Fig. 2*C*)(Barr and Garcia, 2006). It is proposed that they sense the general area of vulva, and promote rhythmic spicule thrusts. Males that had the hook structure precursor cells ablated could not sense the area of vulva and passed the vulva repeatedly while scanning the cuticle. These males, however, periodically thrust their spicules at random areas of the hermaphrodite cuticle, suggesting that the p.c.s. can promote spicule thrusts independently (Liu and Sternberg, 1995; Barr and Sternberg, 1999; Garcia et al., 2001). The p.c.s. contains three pairs of neurons, PCAs, PCBs, and PCCs (Fig. 2*C*). Evidence suggests that they sense the precise location of the vulva, and

also induce shallow spicule thrusts. Males with these neurons ablated could stop in the general vulva vicinity but not precisely over it, and they displayed brief spicule prodding behavior when they transiently passed the vulva, probably as a result of hook sensillum activity. When the hook sensillum and the p.c.s. neurons were all ablated, the males showed neither spicule activity nor vulva recognition at all when they passed over the vulva (Liu and Sternberg, 1995; Garcia et al., 2001). This data together suggests that these sensory organs facilitate different aspects of vulva location behavior: the general area recognition and precise location; however, they can partially compensate for each other.

Vulva location and spicule prodding eventually result in prolonged spicule protraction once the vulva slit is breached, leading to vulva penetration (Liu and Sternberg, 1995). Each copulatory spicule contains sensory dendrites from two sensory neurons (**SPD** and **SPV**) and their supporting cells (socket cells and sheath cells), wrapped with spicule cuticle. The cell bodies of SPD and SPV are outside of the spicules and are located in the cloacal ganglia (Sulston et al., 1980)(Fig. 2*C*). Behavior defects were analyzed after laser-ablation of these cells, in order to assess their function in regulating male mating. The SPV neurons are suggested to regulate the timing of sperm ejaculation, as ablating these cells resulted in males that could perform most steps of mating but could not transfer sperms. In contrast, the SPD neurons are more likely to facilitate spicule insertion, even though they do not innervate the spicule protractors (Liu and Sternberg, 1995)(Male Wiring Project).

Spicule movements are caused by the contraction of two muscles that are attached to it: the **dorsal protractor** and the **ventral protractor** (Fig. 2*C*). Contraction of these muscles can either cause the spicule to rapidly thrust shallowly, or to fully protrude out of the cloacal opening, probably depending on different contractile states of these muscles. In addition, two retractor muscles, which are also attached to the spicule, hold the spicule inside of the male tail when the protractors are relaxed (Fig. 2*C*)(Sulston et al., 1980; Lints and Hall, 2009). In addition to muscles that are physically attached to the spicules, a sexually dimorphic muscle called **anal depressor** also facilitates spicule protraction. Although ablating this muscle alone does not obviously affect spicule insertion during male mating, it significantly exacerbates defective spicule protraction caused by ablation of neurons that innervate the spicule protractors (Garcia et al., 2001; Garcia and Sternberg, 2003).

The neurotransmitter acetylcholine (ACh) has been shown to cause contraction of the spicule protractor muscles. Application of ACh agonists or excess of endogenous ACh, can cause males to fully protract their spicules, mimicking the spicule insertion behavior during mating. The endogenous ACh that induces sustained protractor contraction comes from a pair of cholinergic neurons, the **SPC**s (Fig. 2*C*). Laser-ablation of these neurons resulted in males that fail to insert their spicules during mating. These neurons have sensory endings attached to the dorsal protractor muscles, and they directly synapse both dorsal and ventral protractors as well as the anal depressor muscle. It has been shown that ACh released from these neurons can cause prolonged spicule

protraction, probably via activating the ionotropic acetylcholine receptors (AChRs) on the spicule protractor muscles (Garcia et al., 2001).

Interestingly, even though it has been suggested that, the hook sensillum neurons and the p.c.s. neurons can independently initiate rapid spicule thrusts at the vulva and two of the p.c.s. neurons (PCB and PCC) can secret ACh, none of these neurons makes synapse to the spicule protractors, indicating that they indirectly command muscle contraction via other circuit components (Sulston et al., 1980; Garcia et al., 2001)(Male Wiring Project). The p.c.s. neurons seem to also play a role in prolonged spicule protraction as ablating these neurons further impairs the SPC-ablated males' response to endogenous ACh. However, it is not clear how these neurons exert their function in these aspects of mating behavior (Garcia et al., 2001).

For most of the neurons mentioned above, their functions in regulating male mating are determined based on behavioral defects resulting from laser-ablating these neurons in behaving males. It is not known how they command their postsynaptic partners to execute specific steps of mating. The "**copulation circuit**" that facilitates the spicule movements (rapid shallow thrusts and prolonged full protraction) probably is the part of male-specific circuit that has been studied most. However, the functional structure of the copulation circuit is still far from being fully understood. In other words, it is not known how sensory information is processed and integrated to eventually command different types of muscle contractions, how the circuit coordinates these motor outputs in a proper sequence, and what molecules shape the synaptic transmission in this

circuit to support the functional connectivity. In this dissertation, I will address these questions.

Study various aspects of behavioral regulation using the *C. elegans* male mating as a model

The complexity of *C. elegans* male mating behavior provides great potential for studying various aspects of behavioral regulation, which will be elaborated in the next few paragraphs. However, the relative lack of understanding of the organization of the male circuits is incompatible with the fact that much attention has already been drawn to study different aspects of the behavior. For example, neurons and muscles in these circuits must be under modulation of various environmental and internal factors; however, without an understanding of how excitable cells in the circuit are used to generate and regulate behavior, it is difficult to interpret defective phenotypes caused by disrupting molecular components of the modulatory mechanisms. Therefore, studying the basic operational logic of the copulation circuit will elucidate the neural substrates for various study topics, such as modulatory mechanisms of behavior, evolutionary basis of different behavioral patterns in reproduction between closely related nematode species, and behavioral differences caused by sex dimorphism.

Modulation of sex muscle excitability by external environmental and internal metabolic changes

The *C. elegans* male mating behavior is a motivated behavior, and it is subjected to modulation by external environment and internal metabolic conditions. It has been shown that both chemosensory and mechanosensory perception of food, food ingestion, and the aging process can affect a male's ability to mate (Gruninger et al., 2006; Gruninger, 2008; Gruninger et al., 2008; Garcia, 2010). A question arises from these observations: what molecular signaling pathways are recruited by these factors to modulate the copulation circuit activity?

For signaling pathways that are susceptive to these factors, one of the ways for them to affect mating behavior is to modulate the spicule protractor muscles excitability. Starvation-induced suppression of sex muscle excitability has been studied in the greatest detail. In these studies, a null allele of the ERG-like K^+ channel gene, *unc-103(0)*, causes ~30% of the males to spontaneously protract their spicules constitutively in the absence of hermaphrodite cues ("prc" phenotype for "protraction constitutive"), probably due to elevated protractor muscle excitability (Garcia and Sternberg, 2003). This "prc" phenotype can be suppressed by overnight starvation, or by mutations that interfere with pharyngeal muscle function, suggesting a hard-wired link between food availability and mating behavior (Gruninger et al., 2006). It was found that the sex muscle excitability is modulated via both food sensation and ingestion, since presence of non-digestible bacteria food, which still releases chemical odors, can partially suppress

the “prc” phenotype in *unc-103(0)* males. The odors of food are sensed by the chemosensory neuron, AWC, in the male head. This neuron is constitutively active in the absence of food. Active AWC down-regulates muscle excitability via the insulin-like receptor, DAF-2, on these muscles. Activated DAF-2 receptor has been well studied for its role in regulating life-span, developmentally arrested dauer stage and fat storage (Vowels and Thomas, 1992; Kenyon et al., 1993; Kimura et al., 1997). In these cases, it inhibits the FOXO transcription factor DAF-16 from entering the nucleus, and therefore upregulates regulates transcription of downstream genes (Vanfleteren and Braeckman, 1999; Mukhopadhyay et al., 2006; Braeckman and Vanfleteren, 2007). However, DAF-16 is not required for DAF-2 to suppress sex muscle excitability, instead, DAF-2 acts through PLC-γ to activate downstream CaMKII signaling in the male sex muscles (Gruninger et al., 2008). The activated CaMKII, during starvation, upregulates activity of the *ether-a-go-go* (EAG) K^+ channel EGL-2 (Weinshenker et al., 1999), and thus lower the sex muscles excitability (LeBoeuf et al., 2007).

Nevertheless, the spicule protractor muscles are just one of the sites of action for modulating male copulation. Molecules, such as CaMKII and UNC-103, are also expressed in neurons in the male copulation circuit (LeBoeuf et al., 2007; Gruninger et al., 2008). This suggests the neuronal components of the circuit can be also under regulation of the signaling pathways reviewed above, and so are other muscles. Consistent with this idea, to rescue the “prc” phenotype in mutant males that carry a loss-of-function allele of the CaMKII gene, CaMKII function needs to be restored not only to the male sex muscles but also to other muscles. This suggests that the CaMKII

signaling is also utilized in other muscles to regulate male mating (LeBoeuf et al., 2007). In *C. elegans,* measuring neuronal or muscular activity directly via electrophysiological methods requires expertise due to the small size of the worm and their neurons (Francis and Maricq, 2006). Therefore, most studies of behavioral regulation are conducted in behaving animals without invasive measurement. However, it is an intimidating task to study modulatory signaling targeted to those circuit components without knowing the behavioral output of their activities. With the understanding of how these neurons regulate the behavior revealed in this dissertation, modulation of the circuit at the different levels can be studied.

Different reproductive behavioral patterns between closely-related nematode species

The comparative analysis of *C. elegans* and the two nematode species that are closely related to it, *C. remanei* and *C. briggsae*, provides a great opportunity to study conserved molecular mechanisms that regulate neural development and behaviors of these animals. More importantly, comparative analysis between closely related nematode species also allows for an understanding of how diverged physiology underpins functional changes among these species through evolution (Coghlan et al., 2006). Molecular genetics has been well studied in *C. elegans*, and it has also started to be studied in the other two species (Lin et al., 2009; Seetharaman et al., 2010; Zhao et al., 2010). In addition, all three genomes have been sequenced and at least partially annotated, and they appear to be very similar (Stein et al., 2003; Hillier et al., 2007;

Barriere et al., 2009). These advantages enable us to explore how proteins evolve among species to build diverse functional structures or determine distinct physiology of the neuronal circuits and eventually give rise to diverged behavioral patterns.

With most studies currently focusing on diversity of gene families and functional molecules among these species, less is known about how neuronal circuits evolve to generate different patterns of behavior (Robertson and Thomas, 2006; Artieri et al., 2008; Haerty et al., 2008; Srinivasan et al., 2008). All these species are morphologically identical, have identical habitat, and display similar behaviors. Surprisingly, males from different species have diverged mating behavior, probably due to the different reproductive needs of individual species. The males of the hermaphroditic species *C. briggsae* mate in a way similar to *C. elegans,* which has been described earlier. *C. remanei* is a gonochoristic species, in which the females need to mate with the males to have progeny. Therefore, as the females have to mate with males to reproduce, they are behaviorally receptive of males' attempts to mate. Once a *C. remanei* male locates a female's vulva with his tail, the female instantaneously stops locomotion, defecation, and pharyngeal pumping, and her vulva slit widens. The soporific behavior and vulva dilation displayed by the female is likely induced by factors secreted by the male, and this enables the male to insert his spicules immediately and then transfer sperm. Except for being more efficient in spicule insertion, the *C. remanei* males execute all other steps of mating similarly to the *C. elegans* males. Interestingly, when these males were set to mate with non-receptive hermaphrodites from the other species, they could not sustain vulva contact once they failed to insert in the first attempt (Garcia et al., 2007). This

indicates that the *C. remanei* males might not have the ability to stay in contact with vulva without spicules being inserted as an anchor. When *C. elegans* evolved independently from the gonochoristic ancestors (Braendle and Felix, 2006), the hermaphrodites lost the mechanism to respond to the male factor and became uncooperative to the males. It is possible that to adapt to the situation, the male then adopted a strategy to maintain prolonged vulva contact. Better understanding of the molecular and cellular mechanisms that regulate each step of the *C. elegans* male mating will enable us to study what changes, on the molecular and cellular level, are necessary to occur in the nervous system for new patterns of behavior to emerge.

Genetic control of sex differences in C. elegans behavior

Like *D. melanogaster*, *C. elegans* has been used as a model organism to study sex differences in nervous system structure and function (Portman, 2007). Different from vertebrates, where the sexual differentiation heavily relies on the gonadal steroid hormones, the *C. elegans* sex difference is cell-autonomously regulated by the master gene *tra-1* (Hodgkin, 1986). *tra-1* encodes a transcription factors and is turned on in hermaphrodites and off in males, as a result of a signaling cascade that assesses the sex chromosome-to-autosome ratio (Zarkower and Hodgkin, 1992; Portman, 2007). Previous research has shown that nearly all sexually dimorphic cell fates are determined by *tra-1*. Several downstream effector molecules have been identified to determine the cell-lineage, programmed cell death, and development of sex-specific tissues (Hunter and

Wood, 1990; Portman, 2007). However, it remains largely unknown what signaling hierarchy links *tra-1* to sex-specific behaviors.

Mating is the major sex-specific behavior for *C. elegans* males. It is an ultimate result of sex-specific differentiation of the nervous system. The male has 89 sex-specific neurons, which are probably responsible for generating the mating behavior (Sulston et al., 1980). A couple of genes have been identified as downstream effectors of *tra-1* to regulate the sexual phenotype of the male-specific nervous system (Portman, 2007). For example, DM-domain genes, such as *mab-23, mab-3* and *dmd-3*, have been shown to regulate development of the male-specific tissues (rays, sex muscles, male tail hypodermis) that are obviously required for male mating (Shen and Hodgkin, 1988; Lints and Emmons, 2002; Mason et al., 2008). However, sex difference is not limited to differences in morphology or structural development that are obvious under the microscope. Differences also exist in mechanisms that determine the circuit physiology or define the connectivity between excitable cells (Lee and Portman, 2007). To understand the mechanisms that determine this type of difference, an understanding of how molecular mechanisms shape physiology of the functional components of the male circuitry and how that gives rise to behavior is required.

Dissertation objectives

The objective of this dissertation is to elucidate cellular and molecular components that are necessary for a neuromuscular circuit to generate complex motor

behavior for an animal to reproduce. The circuit studied is the cholinergic *C. elegans* male copulation circuit. This dissertation will address how activities of different male-specific neurons and muscles generate distinct and coordinated behavioral patterns to facilitate male copulation. It will also showcase that, with the newly gained knowledge of functional circuit connectivity, a G-protein coupled signaling pathway which I uncovered in an earlier study, can be put into a better context to modulate the behavior. This work will provide fundamental knowledge about the behavioral circuit connectivity and organization, which will serve as the backbone for future study of functional molecules that regulate the circuit physiology and shape the pattern of male mating behavior in *C. elegans*.

In Chapter II, detailed materials and methods that are used in experiments performed in Chapters III, IV and V are provided. In Chapter III, I demonstrate that the male mating is optimized by a modulatory signaling pathway. This pathway, mediated by the G-protein-coupled mAChR GAR-3, enhances excitability of the male copulation circuit. As a result of activating this pathway, the ionotropic AChR signaling in the circuit is upregulated before and during mating. Hence, loss of GAR-3 function results in males that have reduced efficiency of mating. In Chapter IV, I explore the role of ionotropic AChRs in the copulation circuit, in addition to their previously-described role in promoting spicule protraction. I find the LEV-sensitive AChR and the α7-like ionotropic AChR are used to mediate neurotransmission between the postcloacal sensilla (p.c.s.) neurons and posterior male tail muscles. Contraction of these muscles is required for the male to maintain his tail position at the hermaphrodite's vulva. In Chapter V, I

address the mechanism the circuit employs to regulate the bistable states of the spicule protractor muscles, rapid shallow contraction and prolonged full contraction. I demonstrate that rapid shallow protractor contraction is induced by the p.c.s. neurons, however, not via direct synaptic transmission. The activated posterior male tail muscles relay signal molecules to the protractors through gap junctions, and then cause regenerative Ca^{2+} current to induce protractor contractions. I also determine that for the protractor muscles to switch to prolonged full contraction, the copulation circuit needs to integrate multiple sensory signals, including precise contact between a male's cloaca and a hermaphrodite's vulva and partial penetration of the vulva slit. An ERG-like K^{+} channel UNC-103 is utilized by the circuit to maintain the lower circuit excitability, so that presence of fewer signals results in lower probability of spicule protraction.

CHAPTER II

EXPERIMENTAL PROCEDURES*

Strains

All strains were grown at 20°C on nematode growth media (NGM) plates seeded with *E. Coli* OP50 (Brenner, 1974). Pharmacological and behavioral assays were conducted between 21-23°C. All males contained *him-5(e1490)* on linkage group V(LGV) (Hodgkin et al., 1979). Additional alleles used were: *unc-29(e193)* (Lewis et al., 1980a; Kim et al., 2001), *unc-38(sy576)* (Garcia et al., 2001), *egl-30(ad805)* (Brundage et al., 1996), *egl-30(tg26gf)* (Doi and Iwasaki, 2002), *unc-29(e1072)* (Lewis et al., 1980a), *unc-73(ce362)* (Williams et al., 2007) on LGI; *unc-103(n1213)* (Park and Horvitz, 1986), *pha-1(e2123)* (Schnabel and Schnabel, 1990), *unc-64(e246)* (Brenner, 1974) on LGIII; *cha-1(p1152)* on LGIV (Rand and Russell, 1984); *unc-17(e245)* (Brenner, 1974), *egl-8(n488)* (Miller et al., 1999) on LGV, *acr-16(ok789)* and *gar-3(gk305)* on LGV were generated by the *C. elegans* Gene Knockout Consortium; *unc-9(e101)* (Brenner, 1974) and *lite-1(ce314)* on LGX (Edwards et al., 2008). Alleles used in this dissertation are summarized in Table A-2.

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* Part of this chapter is reprinted with permission from "G{alpha}q-Coupled Muscarinic Acetylcholine Receptors Enhance Nicotinic Acetylcholine Receptor Signaling in Caenorhabditis elegans Mating Behavior" by Liu Y, LeBoeuf B, Garcia LR, 2007, *Journal of Neuroscience*, 27, 1411 – 1421. Copyright © 2007 by Society for Neuroscience.

The *sy576* mutation in *unc-38* creates a C to T missense mutation that causes the sequence ttgttcccgtttgat to change to tggttcctgtttgat. The proline to leucine change occurs in the di-cysteine loop of the protein. This allele was isolated by Dr. Rene Garcia.

Behavior assays

Assay for spontaneous spicule protraction (Prc) phenotype

Males at the L4 larval stage were separated from hermaphrodites and put onto a fresh NGM agar plate containing *E. coli* OP50. ~20 hrs later, adult virgin males were scored for spontaneous spicule protraction. Males with spicules partially or fully protracted for more than 10 seconds were scored positive for the Prc phenotype.

Observation of behaviors on Oxo M plates

0.5 ml of 100 mM Oxo M was applied on top of NGM plates containing an *E. coli* OP50 lawn. When the drug had soaked into the agar, 5-10 virgin adult males were placed onto the OP50 lawn. Behaviors were then observed with a Zeiss Stemi SV 11 microscope.

Mating potency assay

Males were separated from hermaphrodites at the L4 stage and put onto a fresh OP50-containing NGM plate. The next day, each male was put onto an individual plate

containing a "mating lawn" of OP50 with one free-moving *pha-1(e2123)* hermaphrodite. 25 µl of a saturated OP50 culture was seeded on each plate the previous night. Males and hermaphrodites were incubated together for 4 hours at 20°C, after which the males were picked off. Hermaphrodites were then incubated at 25°C, and their progeny was scored three days later. Homozygotes of *pha-1(e2123)* cannot survive beyond the L1 stage at 25°C (Schnabel and Schnabel, 1990). Plates with L2 or older progeny indicated that cross fertilization occurred. The percentage of males that can sire progeny within 4 hours was recorded for each strain. The wild-type potency was arbitrarily set to 100%, and mating potency of mutants was calculated proportional to the wild type.

Mating observation

L4 males and *unc-64(e246)* L4 hermaphrodites were picked onto separate plates 14 hrs before observation. To make the bacterial lawn used for mating observations, I grew OP50 in LB at 37^{o} C overnight without aeration, concentrated 1 ml of culture via centrifugation and resuspended the bacterial pellet in 20 µl of LB. I then placed ~10 µl of the concentrated bacteria onto the center of the NGM agar plate. After the excess liquid soaked into the agar, I placed 15-30 young adult (1-day into the adulthood) *unc-64(e246)* hermaphrodites and one virgin adult male onto the lawn. Male mating behavior was observed with a Zeiss Stemi SV 11 microscope.

I recorded how long the male spent performing each step of mating behavior using a computer and a time recording Visual Basic Macro written in Microsoft® Excel, which was provided by Dr. Rene Garcia:

```
Sub Macrotimerecorder()

Dim row As Integer
Dim InputVal As String

InputVal = ""
row = 1
Sheets("Sheet1").Activate
Columns("A:B").Select
Selection.ClearContents

Do While (InputVal <> "E")
InputVal = InputBox("Enter S, 1, 2, or E")
If InputVal = "e" Then
   InputVal = "E"
   End If
If InputVal = "s" Then
   InputVal = "S"
   End If
    Cells(row, 1).Value = InputVal
    Cells(row, 2).Formula = "=NOW()"
    Cells(row, 2).Select
    Selection.Copy
    Cells(row, 2).Select
    Selection.PasteSpecial Paste:=xlValues, Operation:=xlNone, SkipBlanks:= _
       False, Transpose:=False
    Application.CutCopyMode = False
    row = row + 1

Loop
End Sub
```

I measured the duration and number of times a male: backed along the length of hermaphrodite searching for the vulva, located and rhythmically prod the vulva with his spicules, moved off the vulva or fully inserted his spicules, ejaculated, retracted his spicules and moved off the hermaphrodite. After the male finished mating, I removed both the male and his hermaphrodite mate. A fresh virgin male was used for each observation.

Vulval contact assay

Males and hermaphrodites were separated from each other at the L4 stage, and put onto separate OP50-containing plates. The next day, 20-30 paralyzed hermaphrodites were put on a mating lawn. One male was then put onto the mating lawn and observed with a Zeiss Stemi SV 11 microscope. For the vulval contact assay in Chapter IV and V, the male behavior was video recorded once he started scanning the hermaphrodite with his tail. 1-day-old adult *unc-64(lf)* hermaphrodites were used to pair with the male. Recordings were stopped 5 minutes later or when the male inserted his spicules (whichever happened first). I recorded how long a male spent performing each step of mating behavior using the aforementioned time recording Visual Basic Macro.

The vulvas of hermaphrodites at this age were not dilated due to extended egg-laying, thus, the vast majority of the males tested in this work could not penetrate the vulva for the duration of the first vulval contact (77.3% of wild type, n=66; 78.9% of *unc-29(lf)* locomotion-restored males, n=19; 92.3% of *acr-16(0)* males, n=13; 87.5% of *unc-29(lf);acr-16(0)* locomotion-restored males, n=32; 100% of the SPC-ablated males, n=11). Therefore, the average duration of vulval contact of an individual male was averaged from multiple vulval contacts, and the efficiency of spicule insertion was not likely to affect our measurements of vulval contact duration. Multiple males were analyzed for each strain or treatment. Wild-type males/non-operated males were always observed in parallel with mutant males/operated males as control. Statistic comparisons were only conducted between male samples that were observed in parallel, since the

wild-type behavior can vary on a day-to-day basis. Comparison between two samples was done by using the Mann-Whitney non-parametric test.

Sperm release

The behavior of males was filmed and the duration of sperm "release" was recorded as described earlier. As defined by a previous study, sperm "release" refers to the process when sperm leaves the seminal vesicle and is release from the vas deferens (Schindelman et al., 2006).

Pharmacology

Levamisole (LEV) (ICN Biomedicals, Aurora, OH) was prepared in distilled water and stored at -20°C. 400 µl of LEV solution at various concentrations was added to a Pyrex Spot Plate (nine 1 ml volume depressions), and ~5-10 virgin males were put into the drug. I observed these males for 3 minutes under a Leica MZ 7.5 stereomicroscope. A male was considered sensitive to the drug if he fully protracted his spicules for > 5 seconds. Fresh drug was used after ~30 virgin males were assayed. Comparisons were made by using the Fisher's exact test.

Oxotremorine M (oxo M) (Sigma-Aldrich, St. Louis, MO) was prepared and applied using the same methods as LEV. A male was considered sensitive to the drug if he protracted his spicules within a 10-minute observation window, since males respond to oxo M not as rapid as to LEV.

Plasmids

Expression pattern of gar-3, acr-16, unc-6, unc-38, unc-29 and acr-18

Primer sequences are provided in Table A-1. A 6 kb of DNA upstream of *gar-3* ATG was PCR amplified, using primers ATTB1gar-3Aup and ATTB2gar-3Adwn (Table A-1), to analyze the *gar-3* expression pattern driven by the promoter P*gar-3*A as reported by Steger and Avery (Steger and Avery, 2004). A 5.3 kb region upstream of the *acr-16* ATG was PCR amplified with the following primers: ATTB1acr-16 and ATTB2acr-16 (Table A-1). A 2.4 kb region upstream of the *unc-63* ATG, plus the first three codons, was PCR amplified with the following primers: attb1unc-63 and unc-63attb2 (Table A-1). A 4.3 kb genomic region that contained 1.2 kb upstream of the *unc-38* ATG and all *unc-38* introns and exons up to the stop codon was PCR amplified with the following primers: attb1unc-38fus and Attb2unc-38fusnew (Table A-1). A 2.2 kb genomic region that contained a 1.3 kb region upstream of the *unc-29* ATG and part of the *unc-29* genomic sequence up to the end of the second exon was PCR amplified with the following primers: ATTB1Punc-29 and ATTB2Punc-29 (Table A-1). A 2.3 kb genomic region that contained 1.2 kb upstream of the *unc-29* ATG and all *unc-29* introns and exons up to the stop codon was PCR amplified with the following primers: ATTB1Punc-29 and ATTB2U29lstex (Table A-1). A 6.2 kb region upstream of the *acr-18* ATG was PCR amplified with the following primers: ATTB1acr-18 and ATTB2acr-18noatg (Table A-1).

All these PCR primers contained Gateway ATTB sites, which allowed the *gar-3A, gar-3B, acr-16*, *unc-63*, *unc-38*, *unc-29* and *acr-18* PCR products to be recombined, using BP clonase (Invitrogen), into the low copy number Gateway entry vector pDG15, to generate pLR56, pLR98, pLR149, pLR125, pYL21 (includes the partial *unc-29* gene), pLR198 (includes the full-length *unc-29* gene) and pLR162, respectively. In order to place the acetylcholine receptor sequences in front of YFP, pLR98, pLR149, pLR125, pYL21 and pLR198 were then individually recombined with the YFP destination vector pGW322YFP (Reiner et al., 2006) using LR clonase (Invitrogen) to make the plasmids pLR59, pLR106, pLR152, pLR127, pYL22 and pLR199, respectively. pGW322YFP and pDG15 have been described in previous studies (Gruninger et al., 2006; Reiner et al., 2006). pLR56, pLR59, pLR98, pLR106, pLR125, pLR127, pLR149, pLR152, pLR162, pLR198, and pLR199 were constructed by Dr. Rene Garcia and Dr. Daisy Gualberto.

To test if the sequence P*gar-3*B was sufficient to facilitate the transcription of *gar-3*, 3.5 kb DNA upstream of the *gar-3* isoform Y40H4A.1a.1 5' UTR was amplified using primers Gar3upstrmRv and Dwngar3B (Table A-1). The P*gar-3*B promoter was blunt-end cloned into the *SmaI* site of the YFP-containing plasmid pSX322 (Reiner et al., 2006) to generate pYL1.

Rescue using gar-3 genomic sequence driven by the hsp16-2 promoter

To drive *gar-3* expression using the heat shock promoter *hsp16-2*, the Gateway Conversion Reading frame Cassette C.1 (Invitrogen) was cloned into the *Eco*RV site of the heat shock promoter vector pPD49.78 (courtesy of A. Fire, Stanford University

School of Medicine, Stanford, CA) (Addgene plasmid 1447, Addgene, Cambridge, MA) to generate the Gateway destination vector pTG14. This construct was made by Dr. Todd Gruninger, a former member in the lab. Primers ATTB1GAR-3strt and ATTB2Gar-3end (Table A-1) were used to amplify 4.9 kb of *gar-3* genomic DNA and flank the DNA with attB sites. Using BP clonase, the PCR product was recombined into pDG15 to generate pYL3. *gar-3* was then recombined from pYL3 into pTG14, using LR clonase, to generate pYL4.

Rescue using gar-3 genomic sequence driven by gar-3 endogenous promoters

A 4.9 kb genomic *gar-3*-containing sequence from the ATG to last asparagine codon was PCR amplified via primers Gar3xbaF and Gar3avrR (Table A-1). To fuse the C-terminal end of GAR-3 to YFP, the PCR product was cut with *Xba*I and *Avr*II, and then cloned into the *Xba*I site of the YFP-containing plasmid pSX322 to generate pYL5. To add different promoters in front of *gar-3::YFP,* pYL5 was cut with *Xba*I and ligated to the Gateway Vector Conversion Reading frame Cassette C.1 (Invitrogen) to generate the Gateway destination vector pYL6. To generate *Pgar-3A: gar-3::YFP*, the *gar-3* promoter P*gar-3*A contained on pLR56 was recombined into pYL6, using LR clonase, to generate pYL8. To construct P*gar-3*B*: gar-3::YFP*, the 3.5 kb *gar-3* promoter P*gar-3*B was PCR amplified via primers ATTB1gar-3Bup and ATTB2gar-3Bdwn (Table A-1) and then recombined into plasmid pDG15, using BP clonase, to generate the entry clone pLR57. pLR57 was then recombined with pYL6, using LR Clonase, to generate pYL9.

Rescue by tissue-specific gar-3 expression

Entry clones pLR21 and pLR28 contain the 5.4 kb *unc-103* promoter P*unc-103E* and the 5 kb *unc-103* promoter P*unc-103F*, respectively (Reiner et al., 2006). The *unc-103* promoters were recombined from pLR21 and pLR28 into the Gateway destination vector pYL6 to generate pYL10 and pYL11.

Cell specific expression of unc-29 cDNA

A SL2-accepting *trans*-splice site was inserted between the *unc-29* cDNA (including the stop codon) and the GFP gene to use GFP flourescence as a proxy for *unc-29* expression. The intergenic region between *gpd-2* and *gpd-3*, containing a SL2-accepting *trans*-splice site, was PCR amplified with the following primers: igrgpd3 and gpd2igr. This region was then cloned into the *Sma*I site of pPD95.69 (Addgene plasmid 1491) to generate pJP1. pJP1 was generated by Jodi L. Bollinger, a former member in the lab. pJP1 was cut with *Hind*III and *Apa*I and then cloned into the *Hind*III/*Ava*I sites of pBR322 to generate pDG4, which contains the SL2 site in front of the GFP gene. The *unc-29* cDNA was then PCR amplified from a cDNA library using the primers Func-29 and unc-29R. The *unc-29* cDNA was cloned into the *Bam*HI site of pDG4 to generate pDG5. pDG5 was then cut with *Xba*I, blunt-ended and then ligated with the Gateway Vector Conversion Reading frame Cassette C.1 (Invitrogen) to generate pYL16. The *acr-8* and the *lev-11* promoter regions were recombined into the entry vector pDG15 to generate pLR92 and pLR22, respectively (Gruninger et al., 2006; Gruninger et al.,

2007). Then I recombined the promoter sequences in pLR92 and pLR22 into pYL16 using LR clonase (Invitrogen) to generate pYL18 and pYL20, respectively.

Cell specific expression of ChR2

To put a Gateway destination cassette situated in front of ChR2, a *Pmyo-3::ChR2(gf)::YFP* plasmid (a gift from Dr. Gottschalk, Goethe University Frankfurt) (Nagel et al., 2005) was cut with *Hind*III and *Bam*HI, blunt-ended and then ligated with the Gateway Vector Conversion Reading frame Cassette C.1 (Invitrogen) to generate pZL5 (made by Dr. Robyn Lints). A TGA stop codon 50 nucleotides downstream of the *Xba*I site was changed to GGA to generate pLR167. To express ChR2 from the promoter region of *acr-18*, *unc-103* (promoter E and F), *lev-11* and *gar-3* (promoter B), pLR162, pLR21, pLR28, pLR22 and pLR57 were subsequently recombined with pLR167 to make pLR165, pLR176, pYL41, pLR178 and pLR183, respectively. Construction of pLR21, pLR22, pLR28 and pLR57 have been described previously (Reiner et al., 2006; LeBoeuf et al., 2007; Liu et al., 2007; Gruninger et al., 2008).

Transgenics

Plasmids were co-injected with pBX1 (P*pha-1:pha-1*) (50 ng/µl) (except for pLR59 and pYL1, pBX1 was injected at 100 ng/µl) into strains that contained the *pha-1(e2123)* allele (Granato et al., 1994). The F1s and their progenies that could survive beyond the L2 stage were kept as transgenic animals, as the *pha-1(e2123)* phenotype

was rescued by pBX1. For strains that did not have the *pha-1(e2123)* allele, GFP or YFP encoded on one of the injected plasmids was used to identify transgenic animals. For all injections, pUC-18 was used to make the final concentration of DNA to 200 ng/µl.

The expression constructs pLR59, pYL1, pLR106, pLR152, pLR127, pLR199 and pYL22 were injected at 50 ng/µl into the *pha-1(e2123); him-5(e1490); lite-1(ce314)* hermaphrodites. Multiple male-specific muscles are located within a small region of the male tail. Sometimes it is difficult to differentiate these muscles when the majority of them are expressing YFP. The expression of pLR165 (P*acr-18:ChR2::YFP)* was located on the plasma membrane and the ER, making it easier to visualize muscle expression in the male tail (Fig. A-1).

To express the genomic DNA of *gar-3* gene by the heat shock promoter *hsp16*-2, an injection mixture containing pYL4 (25 ng/µl), pBX1 (100 ng/µl) and pUC18 (75 ng/µl) was injected into *pha-1(e2123); him-5 (e1490) gar-3(gk305)*. Three lines were obtained; all transgenic lines were rescued upon heat shock. One of these lines, *pha-1(e2123); him-5 (e1490) gar-3(gk305); rg*Ex65 was further analyzed.

To express the genomic DNA of *gar-3* gene by its own endogenous promoter Pgar-3A, an injection mixture containing pYL8 (20 ng/µl), pBX1 (100 ng/µl) and pUC18 (80 ng/µl) was injected into *pha-1(e2123)*; *him-5 (e1490) gar-3(gk305).* Three transgenic lines were obtained, and the line that contained *rg*Ex90 [P*gar-3A::gar-3::YFP*] was further analyzed. To express the genomic DNA of *gar-3* gene by its own endogenous promoter P*gar-3B*, an injection mixture containing pYL9 (50 ng/µl), pBX1 (100 ng/µl) and pUC18 (50 ng/µl) was also injected into *pha-1(e2123)*; *him-5 (e1490)*

gar-3(gk305). The *him-5 (e1490) gar-3(gk305); pha-1(e2123); rg*Ex92 [P*gar-3*B*::gar-3::YFP*] transgenic line expressed brightly fluorescing GAR-3::YFP. When assayed with 50 mM Oxo M, this line was rescued for Oxo M sensitivity. However, after 3- 4 generations, YFP expression became faint and disappeared eventually. Reducing pYL9 concentration to 10 ng/µl and 1 ng/µl in the injection mixture didn't solve this problem. I speculated that the reduced YFP expression might be due to silencing of repetitive transgene arrays. Thus I used *Pvu*II-digested *C. elegans* genomic DNA (50 ng/µl) as the carrier DNA to coinject with pYL9 (50 ng/µl) and pBX1 (100 ng/µl). 15 lines were obtained and YFP expression in all of them was stable. The line *him-5 (e1490) gar-3(gk305); pha-1(e2123); rg*Ex107 was further analyzed. Interestingly, when pYL8 and pYL9 were coinjected at 30 ng/µl, expression from both constructs was stable, even when *Pvu*II-digested *C. elegans* genomic DNA was not used as a carrier. Presumably, some sequence in pYL8 can reduce the silencing of pYL9.

To express the *gar-3* gene using other tissue-specific promoters, 50 ng/µl of pYL10 and pYL11 plasmids were injected separately with pBX1 (100 ng/µl) and pUC18 (50 ng/µl) to generate the extrachromosomal arrays *rg*Ex94 *(*containing P*unc-103E::gar-3::YFP)* and *rg*Ex95*(*containing P*unc-103E::gar-3::YFP).*

To rescue the locomotion defects of *unc-29(lf)* males, pYL18 (70 ng/µl) and pYL20 (20 ng/µl) were injected into *unc-29(e193); him-5(e1490)* or *unc-29(e193); acr-16(ok789) him-5(e1490)* hermaphrodites. To express G-CaMP and DsRed simultaneously in the male-specific muscles and express ChR2 in the oblique-gubernacular muscle group, pLR135 (20 ng/µl), pLR136 (50 ng/µl) and pLR165

(100ng/µl), were injected into the *pha-1(e2123); him-5(e1490); lite-1(ce314)* hermaphrodites by Todd Gruninger. pLR35(P*unc103*E*::G-CaMP*) and pLR36(P*unc-103*E*::DsRed*) are described elsewhere (Gruninger et al., 2008). To express ChR2 in specific tissues, pLR176 (50 ng/µl), pLR178 (50 ng/µl), pLR183 (100 ng/µl) and pYL41 (150 ng/µl) were injected into the *pha-1(e2123); him-5(e1490); lite-1(ce314)* hermaphrodites.

The transgenic line *xu*Ex[P*unc-38::YFP + lin-15(+)*]*; lin-15(n765ts)* was a gift from Dr. Shawn Xu (Life Sciences Institute, University of Michigan). Plasmids containing the *unc-38 promoter::YFP* construct (100 ng/µl) and the *lin-15* gene (40 ng/µl) were co-injected into *lin-15(n765ts)* hermaphrodites. The *unc-38 promoter::YFP* plasmid is a transcriptional fusion that contains YFP expressed from 1.1 kb DNA upstream of the first ATG of *unc-38*.

Laser ablation

Cell ablations were done using the standard protocol (Bargmann and Avery, 1995). The operation was conducted using a Spectra-Physics VSL-337ND-S Nitrogen Laser (Mountain View, Ca) attached to an Olympus BX51 microscope. L2 worms were operated on 5% agar pads containing 0.5 mM NaN_3, and L4 males were operated on 5% agar pads containing 2 mM NaN_3. For each operated animal, a control animal was placed on the same agar pad for the same amount of time to rule out the possibility that behavioral changes are due to the anesthetic pads. Ablation of the PCA and PCB neurons

was done by Brigitte LeBoeuf, and she also collected the data to produce the vulval contact profiles of these animals.

Optical stimulation and detection of repetitive spicule thrusts

lite-1(ce314) males that expressed P*acr-18:ChR2::YFP* were used. At L4 stage, males were transferred onto NGM plates with OP50 supplemented with all-*trans* retinal. Males with the same transgenes were also placed onto NGM plates with regular OP50 as control. All-*trans r*etinal-containing plates were prepared the day before by spreading OP50 culture that contained 50 μM all-*trans* retinal (A.G. Scientific).

The next day, the males were immobilized on 10% agarose (in H2O) pad containing 0.5 μl of 0.1 μm diameter polystyrene microspheres, and covered with a coverglass (Fang-Yen et al., 2009). Sequences of DIC images of the male tails were recorded under an Olympus BX51 microscope. A Dual View Simultaneous Imaging Systems by Photometrics (Surrey, BC) was used and adjusted to split the image signal, so that DIC image could be recorded in one field of view (field 1), and simultaneously, fluorescent signals could be recorded in the other field with a dimmer DIC image (field 2)(Fig. A-2). Multiple blue light pulses were applied manually to each male tested. Once the blue light was turned on, the YFP fluorescent signal was detected in field 2 and this was used to indicate the timing of light stimulation in our recording (Fig. A-2). An ROI was placed in the DIC image in field 1 at the base of one spicule (Fig. A-2). During spicule thrusts, light refractivity of the region defined by the ROI changed, resulting in

changes in the standard deviation of the pixel intensity within this ROI (SDEV) (Fig. A-2). Therefore, the value of SDEV throughout the image sequence was used to indicate the displacement of spicule.

Optical stimulation and Ca^{2+} imaging

Strains used to image Ca^{2+} transient contained *lite-1(ce314)*. Muscular Ca^{2+} transients were measured by detecting changes in fluorescence intensity of G-CaMP. The red fluorescent protein DsRed was expressed in the same set of cells as G-CaMP. Since the fluorescence intensity of DsRed does not change in response to light stimulation, it was used as an internal control. ChR2 is expressed in the gubernacular-oblique muscle group. DsRed and G-CaMP signals were recorded separately, but simultaneously via the Dual View Simultaneous Imaging Systems with an OI-11-EM filter by Photometrics (Surrey, BC). To record G-CaMP and DsRed, the transgenetic males were placed on agar pads without NaN_3, and were observed under an Olympus BX51 microscope using a 40× objective. The images were recorded using a Hamamatsu ImagEM Electron multiplier (EM) CCD camera. Series of pictures were taken at the speed of ~25 frames per second for 1 minute after the blue light was turned on. To record Ca^{2+} changes, each transgenic male was separated from hermaphrodites at the mid-L4 larval stage, and grown on OP50 lawns overnight. The adult male was recorded for the first time with non-functional ChR2, to obtain the baseline ratio of G-CaMP/DsRed intensity (R_0). This ratio does not change within a short period, since G-

CaMP and DsRed expression were under control of the same promoter. Afterwards, they were incubated on OP50 lawns that contain all-*trans* retinal for 30 minutes and then reimaged under blue light.

Laser stimulation and Ca^{2+} imaging

Laser stimulation of muscles was slightly modified from a previous study (Reiner et al., 1995). 18 -24 hr adult males containing G-CaMP and DsRed in their muscles were put on a 10% agarose (in H_2O) pad containing 0.5 µl of 0.1 µm diameter polystyrene microspheres, and covered with a coverslip (Fang-Yen et al., 2009). The minimum output of a Spectra-Physics VSL-337ND-S Nitrogen Laser was adjusted to induce muscle contraction. The laser was aimed at the gubernaculum erector or a posterior body wall muscle, and one to ten pulses were applied to induce the muscles to contract. G-CaMP and DsRed fluorescence changes were then measured in the gubernaculum and body wall muscles, as well as the anal depressor and protractor muscles.

Analysis of Ca^{2+} imaging data

The Hamamatsu SimplePCI (version 6.6.0.0.) software was used to analyze the movies. The region-of-interest (ROI) that covers the spicule protractor and anal depressor muscles was defined manually for both G-CaMP and DsRed, and an ROI was picked far away from the male as the background region for both channels. All ROIs had

the same shape and area (by using the "ROI clone" tool), and the "mean grey" of the ROIs was calculated for each frame as the fluorescence intensity. The intensity of the background region ROI was subtracted from the sample region ROI to exclude background fluorescence from other sources.

The intensity of DsRed does not change in response to Ca^{2+} transients. The average intensity of the DsRed was calculated through the image sequence ($DsRed_{average}$). The intensity of DsRed for each frame ($DsRed_n$) deviates from the "$DsRed_{average}$" only because of photo bleaching and/or changes in muscle shape, which should contribute to G-CaMP signal change as well. To cancel out these Ca^{2+}-unrelated changes in G-CaMP signals, the intensity of G-CaMP in each frame ($G\text{-}CaMP_n$) was then normalized by using the equation of "$G\text{-}CaMP_{normalized\ n} = G\text{-}CaMP_n \times DsRed_{average} / DsRed_n$".

For the baseline recording before all-*trans* retinal incubation, a ratio of "$G\text{-}CaMP_{normalized\ n}/DsRed_{average}$" for each frame was calculated ($R_{0\ n}$), and the average of this ratio within the first 2-4 seconds of recording (R_0) was used as the baseline signal.

For the second recording after all-*trans* retinal incubation, the ratio of "$G\text{-}CaMP_{normalized\ n}/DsRed_{average}$" for each frame was also calculated (R_n). Finally, the Ca^{2+} level change ($\Delta R/R_0$) for this male was then calculated by "$(R_n - R_0)/R_0 \times 100\%$". This ratio is comparable to the $\Delta F/F0$ ratio used in other literature, except for that a G-CaMP/DsRed ratio is used instead of merely the G-CaMP intensity. The ratio $\Delta R/R_0$ was plotted over time as "corrected G-CaMP fold change trace for stimulated recording".

The maximum fold change (max. $\Delta R/R_0$) throughout the frame sequence was then calculated. Finally, the "max. $\Delta R/R_0$" was used as the maximal Ca^{2+} level change for each male, and was plotted and compared between treatments groups using the Mann-Whitney non-parametric test.

Optical stimulation and continuous Ca^{2+} imaging

A demonstration unit of the Mosaic Imaging System (Andor™ Technology) was used to image G-CaMP and DsRed in the protractor muscles before, during and after light-stimulation of the gubernacular-oblique muscle group. The image sequences were taken at a rate of ~72 frames/second. Males that expressed P*acr-18:ChR2::YFP*, P*unc-103E:G-CaMP* and P*unc-103E:DsRed* were used in this assay. Males were immobilized by using the 10% agarose pad and polystyrene microspheres described earlier. Only the region of protractors was illuminated and monitored in the first 100 frames of images (~1.4 seconds). Then, a region containing the gubernacular-oblique muscles was subsequently stimulated with blue light for 1000 frames of recording (~14 seconds). The protractors were continuously monitored during this period, and were recorded for another 500 frames (~6.9 seconds) after the end of gubernacular-oblique stimulation. The Ca^{2+} level change ($\Delta R/R_0$) was then calculated by "$(R_n - R_0)/ R_0 \times 100\%$". R_n and R_0 were determined using methods described earlier. For some males, periodic spicule movements were seen before stimulation of the gubernacular muscles, due to pressure applied on the male tail by the coverglass.

RNA interference

RNAi was induced by feeding worms bacteria producing double stranded RNA to the target genes. Bacteria containing the target genes were obtained from the *C. elegans* ORF-RNAi library (Rual et al., 2004). Bacteria with the L4440 double-T7 vector but with no target gene was used as the negative control. Bacteria were grown and induced by IPTG using a standard protocol (Kamath et al., 2001). L4 males were transferred to plates spotted with the bacteria that express dsRNA and were incubated for ~20 hours. The adult males then were assayed for their response to light stimulation. The target gene sequences in the bacteria were verified by PCR amplification and sequencing using the universal primers (Rual et al., 2004).

Injection of carbenoxolone and tubocurarine

Males that expressed P*acr-18:ChR2::YFP*, P*unc-103E:G-CaMP* and P*unc-103E:DsRed* were injected with the drugs. Carbenoxolone disodium salt (CBX) and (+)-Tubocurarine were purchased from Sigma-Aldrich (St. Louis, MO). Solution of CBX or tubocurarine was injected into the males using the same procedure as DNA microinjection. Males were imaged to obtain the baseline G-CaMP/DsRed ratio, and then were incubated on OP50 supplemented with all-*trans* retinal for 30 minutes. Drug or water was injected right after the incubation. These males were allowed to recover on OP50 supplemented with all-*trans* retinal for 1 hour, before they were imaged again to

assess light-induced changes in the G-CaMP/DsRed ratio. For the injection needle not to stimulate any muscles or neurons in the male tail, one injection pulse was done at 1/3 of body length away from the posterior end of the male, the other was done in the anterior half of the male. A series of different concentrations of solution were injected for each drug to determine the proper concentration for our measurements. 7.5 mM or higher of CBX was found completely to suppress the light-induced repetitive spicule thrusts as well as the locomotion (Fig. A-3). Upon light stimulation, the G-CaMP signal then was measured in males that were injected with 7.5 mM CBX. The effects of different concentrations of tubocurarine were evaluated, and 7.5 mM or higher of tubocurarine was found to completely suppress the worm's locomotion. 10 mM of tubocurarine then was injected for the recordings.

Assay for blue light induced behaviors

All strains used in this assay contained the *lite-1(ce314)* allele. At L4, males expressing the respective transgenes were transferred onto NGM plates with OP50 supplemented with all-*trans* retinal, which was prepared in the same way as described. Males with the same transgenes were also placed onto NGM plates with regular OP50 as control.

In the assay for light-induced sustained spicule protractor contraction, the SPC, PCA and PCB neurons were laser ablated in males at the mid-L4 larval stage. To remove hook associated cells, P10.p and P9.p were ablated at the L2 stage. The P*lev*-

11:ChR2::YFP expressing males were assayed for light-evoked behavior on 5% agar pads without NaN_3. All other strains were assayed for light-evoked behavior on fresh all-*trans* retinal-supplemented OP50 plates, and their control males were assayed on standard OP50 plates. The behavior was observed using an Olympus SZX16 microscope and recorded using a Hamamatsu ImagEM Electron multiplier (EM) CCD camera. Males were illuminated with blue light from the EXFO X-Cite®120PC Q Fluorescence Illumination System, filtered with the SZX2-FGFPA GFP filter (Ex460-495/Em510-550). Males were filmed in the absence of blue light for the first few seconds and then blue light was manually turned on for ~10 seconds. Males that protracted their spicules in response to illumination for more than 5 seconds were counted as sustained spicule protraction positive.

The *unc-103(0)* males occasionally protrude their spicules in the absence of mating stimulation. Eventually, ~30% of the virgin adult males that are separated from hermaphrodites will protract their spicules permanently. I used males whose spicules were not permanently protracted for the blue light stimulation assay. To rule out the possibility that spicule protraction scored was a result of spontaneous activity of the spicule circuit instead of light-stimulated activity, only males that retracted their spicules once the light was turned off were counted as positive for light-induced spicule protraction.

To determine the percentage of males that display rapid spicule thrusts upon blue light stimulation for the *unc-9(lf)* mutant and innexin RNAi strains, males were placed on 5% agar pads without NaN_3, and observed under the Olympus BX51 microscope. The

blue light was manually turned on for ~5 seconds and the males that displayed repetitive spicule thrusts during this period were counted as positive.

CHAPTER III

A MUSCARINIC ACETYLCHOLINE RECEPTOR ENHANCES THE NICOTINIC ACETYLCHOLINE RECEPTOR SIGNALING IN MALE MATING*

$G\alpha_q$ and synaptic transmission are required for ACh agonist-induced spicule protraction

The molecular mechanism of fast transmission required for prolonged spicule protractor contraction has been studied in great detail from previous work. The cholinergic SPC neurons are required for the prolonged spicule protraction, as ablation of these neurons resulted in males that could not insert their spicules during mating while all other behavioral steps appeared intact (Garcia et al., 2001). The *C. elegans* nervous system has been reconstructed from electromicroscopy images of serial sections of the worm body. Thus, physical connections between any two excitable cells in the worms are known (White et al., 1986). According to the reconstruction, the SPC neurons have chemical synapses directly onto the spicule protractor muscles (Sulston et al., 1980). When applied with a cholinesterase inhibitor aldicarb, the wild-type males protracted their spicules within 500 seconds, as a result of the spontaneously-released

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* Data reported in this chapter is reprinted with permission from "G{alpha}q-Coupled Muscarinic Acetylcholine Receptors Enhance Nicotinic Acetylcholine Receptor Signaling in Caenorhabditis elegans Mating Behavior" by Liu Y, LeBoeuf B, Garcia LR, 2007, *Journal of Neuroscience*, 27, 1411 – 1421. Copyright © 2007 by Society for Neuroscience.

endogenous acetylcholine (ACh) accumulating at the synapses. Loss-of-function mutations that abolish cholinergic neurotransmission nearly eliminated males' response to aldicarb. Similarly, the SPC-ablated males took significantly longer time to protract spicules in aldicarb, suggesting that the SPC neurons secrete ACh to cause spicule protraction (Garcia et al., 2001). Ablating the postcloacal sensilla (p.c.s.) neurons also reduced males' response to aldicarb, indicating that these neurons contribute to spicule protraction as well (Garcia et al., 2001). However, there is no evidence showing any of these neurons innervate the protractors (Sulston et al., 1980)(S.W. Emmons, personal communication, 2006).

The fast cholinergic synaptic transmission that causes muscles to contract is mediated by the ionotropic nicotinic acetylcholine receptors (nAChRs) (Changeux et al., 1970; Peper et al., 1982). These channels are pentamers containing five subunits (Numa et al., 1983; Changeux et al., 1984). Opening of the channel allows cations to flow into the cytosol to activate voltage-gated Ca^{2+} channels and cause membrane depolarization (Peper et al., 1982). To gain an understanding of what types of ionotropic AChRs are expressed on the protractors to induce prolong spicule protraction, different ACh agonists were applied to the males to determine which ones can induce this behavior. In *C. elegans*, an ionotropic AChR can be a heteromeric receptor formed by five different α and non-α subunits; or it can be a homomeric receptor formed by five identical α subunits (Jones and Sattelle, 2004). It was found that levamisole (LEV), an anthelminthic used to treat parasitic worm infections, can cause prolonged spicule protraction, and its action requires a heteromeric ionotropic AChR that contains the

UNC-38 α subunit and the UNC-29 non-α subunit. It was suggested that LEV induces spicule protraction via direct activating ionotropic AChRs on the protractor muscles, as expressing functional *unc-38* on these muscles restored the LEV sensitivity to the *unc-38* loss-of-function *(lf)* mutant males (Garcia et al., 2001). Consistent with this finding, I obtained the *unc-38* gene expression pattern in the male, by expressing YFP using the *unc-38* promoter. It was expressed in the spicule protractor muscles, the anal depressor muscle, and the body wall muscles, but not in any neurons associated with the spicule activities (Fig. 3*A*, *B*).

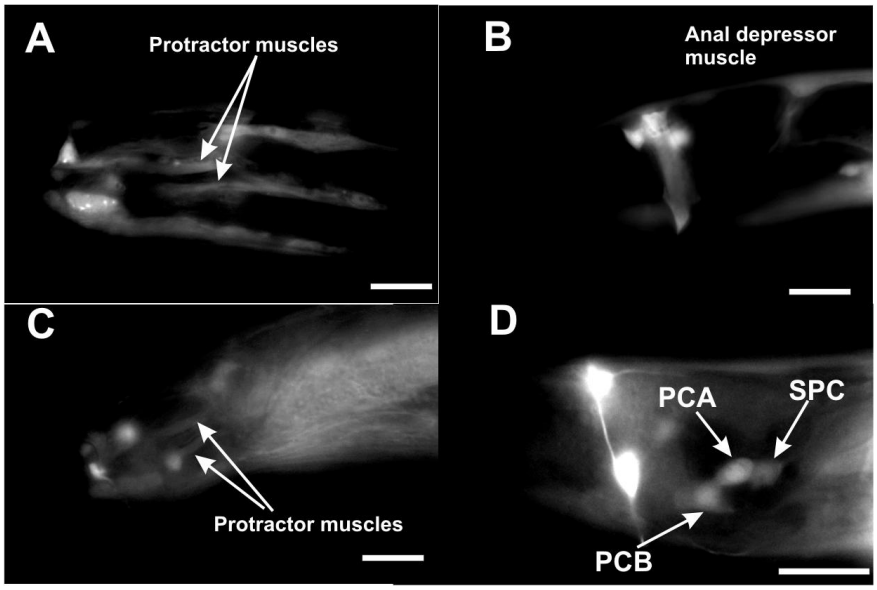

Figure 3. Male tail expression of *unc-38* and *gar-3* promoters.
A. Fluorescence images of the right lateral tail region of an adult male expressing the *unc-38* promoter:YFP construct.
B. Early L4 male expressing the *unc-38* promoter:YFP construct.
C. Adult male expressing the *Pgar-3:YFP* construct.
D. Late L4 male expressing the *Pgar-3:YFP* construct. Scale bar, 10 μm.

Interestingly, it was shown in a previous study that the heterotrimeric G-protein $G\alpha_q$, encoded by the gene *egl-30*, also facilitates the LEV-induced spicule protraction (Garcia et al., 2001). I revisited this observation, and confirmed that for males carrying the *egl-30(ad805)* loss-of-function *(lf)* allele, it takes 12-fold higher concentration of LEV to cause 50% of the population to protract their spicules, compared to the wild type (Fig. 4). G-proteins are usually coupled to the metabotropic receptors on the cell membrane, but not the ionotropic receptors, such as L-AChR. In neurons, the $G\alpha_q$–mediated signaling pathway plays an important role in regulating neurotransmitter release (Lackner et al., 1999; Bastiani et al., 2003; Bastiani and Mendel, 2006). Therefore, I hypothesized that neurotransmission from presynaptic neurons facilitates the LEV-induced spicule protraction.

To test this hypothesis, I asked if mutations that disrupt synaptic transmission would reduce males' response to LEV. The *unc-64* gene encodes the *C. elegans* syntaxin that is required for synaptic vesicle release, and *cha-1* encodes the choline acetyltransferase that is used to synthesize ACh (Alfonso et al., 1994; Ogawa et al., 1998). I assayed mutant males that contain the *unc-64(e246)* loss-of-function allele, as well as mutant males that have the *cha-1(p1152)* loss-of-function allele, for their sensitivity to LEV. When applied with 1 μM LEV, 79% of the wild-type males protracted their spicules (n=85), whereas only 5% of the *unc-64(lf)* males (n=20, $p<0.0001$) and the *cha-1(lf)* males (n=20, $p<0.0001$) responded to the LEV (Fig. 4). This supports that synaptic transmission is required for wild-type LEV sensitivity.

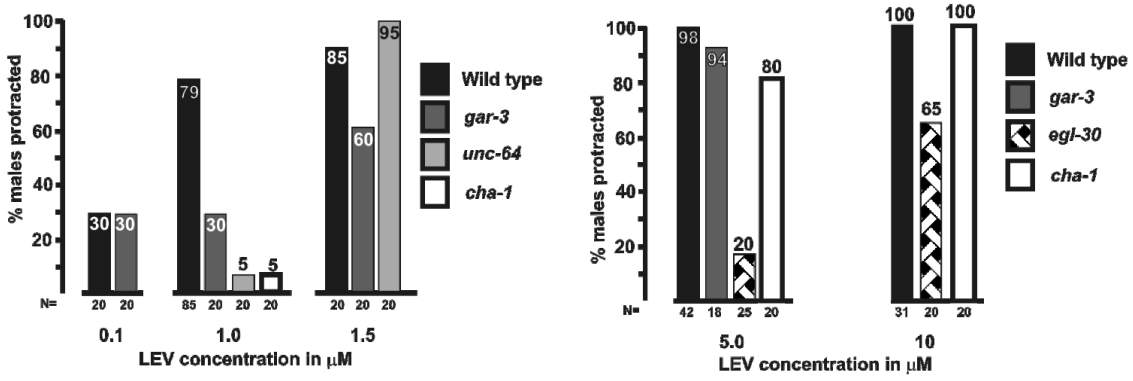

Figure 4. LEV-induced spicule protraction requires GAR-3/Gα_q signaling and cholinergic synaptic transmission.
The number above or within the bars are the percentage of males that protracted spicules in the LEV. The numbers below the bars are the number of males tested.

The SPC neurons are required for spicule insertion and they make synapse to the protractor muscles (Sulston et al., 1980; Garcia et al., 2001)(Male Wiring Project). I then tested if synaptic transmission between the SPCs and the protractors facilitates LEV-induced spicule protraction. I laser-ablated the SPC neurons in males when they were at the L4 larval stage, and found that when they became adults, only 22.2% of the operated males protracted their spicules in 1 μM LEV (n=18), compared to 60% of the control intact males (n=25, $p<0.028$). This, together with the data I showed previously, indicates that Gα_q signaling facilitates LEV-sensitive AChR-induced protractor muscle contraction, via upregulating the cholinergic synaptic transmission between the SPC neurons and the protractor muscles.

A $G\alpha_q$-coupled mAChR, GAR-3, is required for LEV-induced spicule protraction

Loss of $G\alpha_q$ function results in abolished synaptic transmission, whereas a gain-of-function mutation of *egl-30* results in increased ACh release (Lackner et al., 1999; Doi and Iwasaki, 2002). I reasoned that an *egl-30(tg26)* gain-of-function (gf) allele should increase the incidence of spicule protraction, since the spontaneous ACh secretion is upregulated in the mutant worms (Lackner et al., 1999). Indeed, 76% of the *egl-30(gf)* males protract their spicules spontaneously ("prc" phenotype) in absence of any agonists (n=97, Table 1).

To verify whether the "prc" phenotype of the *egl-30(gf)* males is a result of increased cholinergic synaptic transmission, I assayed the *egl-30(gf);unc-64(lf)* and the *egl-30(gf);unc-17(e245)* double mutants. The *unc-17(e245)* loss-of-function allele affects the synaptic vesicle acetylcholine transporter gene (Alfonso et al., 1993). I found both double mutants displayed less incidence of spontaneous spicule protraction (Table 1), suggesting that hyperactivated $G\alpha_q$ causes spicule protractor contraction via upregulating ACh release. Therefore, I conclude that the $G\alpha_q$ signaling is utilized by the spicule circuit to promote spicule protraction.

Table 1. Suppression of the *egl-30(tg26)*-induced Prc phenotype

Genotype	% males protracted spontaneously	n	p-value
egl-30(tg26gf)	76	97	
wild type	7	100	<.0001
egl-30(tg26gf); unc-64(e246)	22	275	<.0001
egl-30(tg26gf); unc-17(e245)	15	27	<.0001

p-values calculated using Fischer's exact test, relative to *egl-30(tg26gf)*.

In *C. elegans,* $G\alpha_q$/*egl-30* is expressed in many excitable cells (Lackner et al., 1999; Bastiani et al., 2003), and it is coupled to various types of membrane receptors to regulate cell physiology in response to different intercellular signals (Bany et al., 2003; Bastiani et al., 2003; Moghal et al., 2003; Bastiani and Mendel, 2006). To study how $G\alpha_q$/*egl-30* signaling specifically regulates the spicule activity, I asked what receptor is coupled to $G\alpha_q$ in the spicule circuit. In a previous study, ACh agonists could induce spicule protraction, whereas agonists of other major neurotransmitters did not (Garcia et al., 2001). I hypothesized that muscarinic acetylcholine receptors (mAChRs), which are G protein-coupled metabotropic ACh receptors, could be coupled to $G\alpha_q$ to promote spicule protraction. To test this hypothesis, I asked if mAChRs agonist can induce spicule protraction, similarly to the *egl-30(gf)* mutation. Arecoline is a mAChR agonist that has been reported to activate $G\alpha_q$ signaling in *C. elegans* to modulate the pharyngeal pumping behavior (Brundage et al., 1996). This drug is also able to induce spicule protraction. However, functional $G\alpha_q$ is not required for this behavioral response (Garcia et al., 2001), suggesting that arecoline also stimulates $G\alpha_q$-independent signaling to promote spicule protraction. Instead, I used another agonist, oxotremorine M (oxo M), which has been used as a non-specific mAChRs agonist in the mammalian system (Freedman et al., 1988; Kaneda et al., 1993; Tayebati et al., 1999; Mistry et al., 2005). I found this drug could induce spicule protraction in wild-type males (Fig. 5). In contrast to arecoline, oxo M acts specifically through the $G\alpha_q$ signaling to induce spicule protraction. In 50 mM oxo M that can cause 90% of the wild-type males to protracted their spicules (EC_{90}) (n=72), only 7% of the *egl-30(lf)* males protracted their spicules

(Table 2, n=30, p<0.0001). This suggests that oxo M is a $G\alpha_q$-specific agonist in the *C. elegans* spicule circuit.

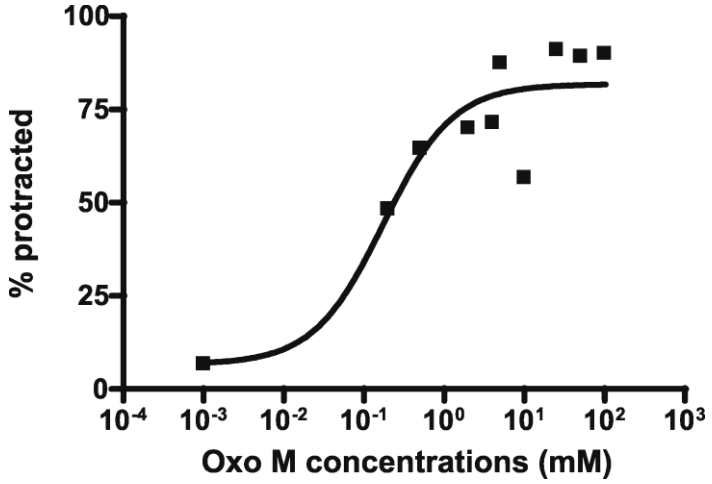

Figure 5. Dose-response of wild-type males to oxotremorine M.
Numbers on the X-axis represent the concentrations of Oxo M at which the wild-type males were treated. Numbers on the Y-axis represent the percentage of males that protracted their spicules within 10 min. Black squares represent the percentage of males that protracted their spicules. About 30 males were assayed for each drug concentration.

To determine if oxo M causes spicule protraction via upregulating the cholinergic synaptic transmission, I tested the *unc-64(lf)* and *unc-17(lf)* mutant males for their ability to protract spicules in oxo M at the EC_{90} concentration. Only 43% of *unc-64(lf)* males (n=30, p<0.0001) and 33% of *unc-17(lf)* males (n=30, p<0.0001) protracted their spicules in the drug (Table 2), suggesting that intact ACh secretion is required for the wild-type response to oxo M. In addition, the *unc-38(lf) unc-29(lf)* double mutant males also had decreased incidence of spicule protraction in the drug: only 58% of them

Table 2. Efficiency of oxotremorine M- induced spicule protraction

Genotype	% males protracted in 50mM Oxo M	n	[a]p-value
wild type	86	72	
unc-64(lf)	43	30	<0.0001
unc-17(lf)	33	30	<0.0001
egl-30(lf)	7	30	<0.0001
egl-8(lf)	65	40	0.015
unc-38(lf) unc-29(lf)	58	31	0.0037
gar-3(lf)	4	50	<0.0001
unc-73(lf)	37	35	<0.0001
unc-73(lf);egl-8(lf)	12	75	<0.0001
SPC-ablated wild type	11	19	<0.0001
PCB-ablated wild type	30	20	<0.0001
SPC- and PCB-ablated wild type	28	67	<0.0001
[b]*gar-3(lf)*	3	34	
[b]wild type	63	87	<0.001
[b]*gar-3(lf); rg*Ex90(P*gar-3*A::*gar-3* (+))	10	20	
[b]*gar-3(lf)*; *rg*Ex92(P*gar-3*B::*gar-3* (+))	62.5	32	<.0001
[b]*gar-3(lf)*; *rg*Ex99(P*gar-3*A + P*gar-3*B::*gar-3* (+))	89	38	<.0001
[b]*gar-3(lf); rg*Ex94(P*unc-103*E::*gar-3 (+))*	92	13	<.0001
[b]*gar-3(lf); rg*Ex95(P*unc-103*F::*gar-3* (+))	97	29	<.0001
[b]*gar-3(lf)*; *rg*Ex65(P*hsp-16-2*::*gar-3* (+)			
- heat shock	9	32	
+ heat shock	100	22	<.0001

[a]p-values were calculated using Fischer's exact test. Non-transgenic males were compared to non-operated wild type. Males that contained *pha-1(e2123)* were compared to *pha-1(e2123);gar-3(lf)*.

protracted spicules in the EC_{90} concentration of oxo M (Table 2; n=31, p<0.0001). The *unc-38* and *unc-29* genes encode different subunits in the same LEV-sensitive AChR (L-AChR) (Ballivet et al., 1996; Fleming et al., 1997; Richmond and Jorgensen, 1999; Garcia et al., 2001; Rayes et al., 2007). This indicates that increased synaptic

transmission promotes spicule protractor contraction partly through activating the L-AChR on the protractor muscles.

Three mAChRs genes are found in *C. elegans*, GAR-1, GAR-2 and GAR-3. They all have similar amino acid sequences as the known mammalian mAChRs M1, M2, M3, M4 and M5 (Hwang et al., 1999; Lee et al., 1999; Lee et al., 2000). Interestingly, in contrast to mammalian mAChRs, only GAR-3 was shown to be sensitive to oxo M, but not GAR-1 and GAR-2. Based on cell culture studies, GAR-3 is coupled to $G\alpha_q$ to activate downstream effector pLCβ (Hwang et al., 1999; Lee et al., 1999; Lee et al., 2000; Park et al., 2000; Park et al., 2003; Park et al., 2006). The *gar-3(gk305)* allele contains a deletion that removes two exons of the gene and generates a premature stop codon in front of the last exon (http://www.wormbase.org; stable release WS160) (Fig. 6). I found that similar to the *egl-30(lf)* mutant, only 4% of the *gar-3(lf)* males protracted their spicules in oxo M at the EC_{90} concentration (Table 2). It suggests that in *C. elegans,* oxo M is a GAR-3-specific agonist in inducing spicule protraction; in addition, oxo M-activated GAR-3 is coupled to $G\alpha_q$ to activate downstream effectors. Consistent with this, the *gar-3(lf)* males also displayed decreased sensitivity to LEV, similar to the *egl-30(lf)* animals. Compared to wild type, the LEV EC_{50} concentration of *gar-3(lf)* males was six times greater (Fig. 4). Therefore, via studying the mAChR GAR-3's role in the spicule circuit, I can study how $G\alpha_q$-signaling is used to regulate the spicule activities.

To rule out the possibility that the *gar-3(lf)* mutation causes oxo M resistance by disrupting development of the excitable cells required for spicule protraction, I asked if

expressing *gar-3* transiently in adult *gar-3(lf)* mutant animals could restore their oxo M sensitivity. I expressed the *gar-3* transcript in adult animals using the *hsp-16-2* heat shock promoter (Stringham et al., 1992). Since development of neurons and muscles that are used for spicule insertion occurs during the L3 and L4 larval stages, the transient expression should not be able to rescue any possible developmental defect. I heat-shocked the adult *gar-3(lf)* males that carried the transgene at 32°C for three hours to induce *gar-3* expression, and found that 100% of these males protracted their spicules in 50 mM oxo M (EC_{90}) (Table 2; n=22, $p<0.0001$). In contrast, only 9% of the control males respond to the drug (Table; n=32). This suggests that the $G\alpha_q$-coupled GAR-3 signaling is used to give rise to animal behavior.

Interestingly, spicule protraction is not the only behavior that is regulated by the GAR-3(mAChR)/$G\alpha_q$ signaling, other aspects of the male mating are also sensitive to oxo M stimulation. Within five to ten minutes of being placed on NGM agar plates containing 5 mM oxo M, 100% of the wild-type males (n=20) displayed behaviors that are normally seen during mating with hermaphrodites. These males initiated backward movements more often when they were crawling, and had a higher tendency to touch themselves or other males using their tails. Additionally, males produced spontaneous dorsal or ventral tail curling and displayed spicule protraction behavior. In contrast, the oxo M-induced behaviors were totally absent in the *gar-3(lf)* males (n=20). This indicates that the GAR-3(mAChR)/$G\alpha_q$ signaling also functions in other components in the circuit that regulates male mating. To my surprise, wild-type hermaphrodites did not show any detectable behavioral change under the same condition, suggesting that the

male-specific circuit is more sensitive to oxo M stimulation. This might be due to differences in GAR-3 receptor membrane expression levels, or due to the intrinsic physiological differences between the male circuit and hermaphrodite circuit.

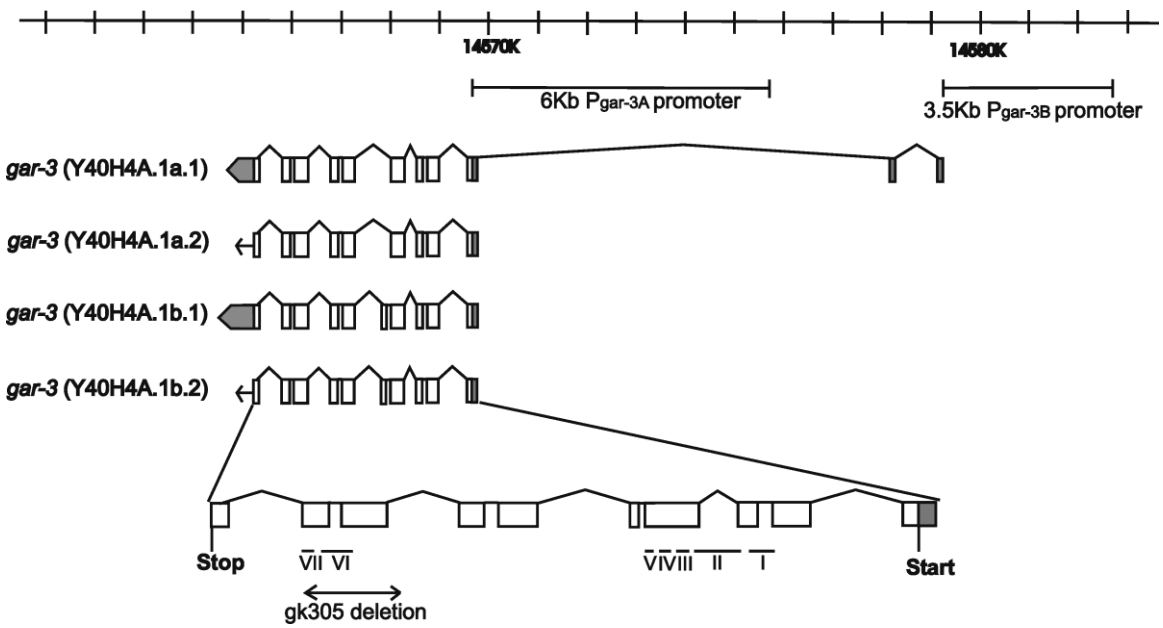

Figure 6. Locations of the *gar-3* promoters and the *gk305* deletion.
The genomic position of *gar-3* is indicated on top; scale bar in kilobases. Two promoters used in this study, P*gar-3A* and P*gar-3B*, are also depicted. Four transcriptional isoforms of *gar-3* are also shown (adapted from Hwang, *et al.* 1999, and Wormbase website, stable release WS160, 2006). Open boxes are exons, grey boxes are UTRs and lines are introns; arrows and arrows merged with grey boxes depict the translation directions. The deletion in the *gar-3(lf)* allele is indicated by a double arrowed line, and the positions of putative transmembrane domains (I-VII) are indicated with horizontal lines (adapted from Park *et al*., 2003).

GAR-3 promotes spicule protraction through phospholipase Cβ and Trio Rho-GEF

Phospholipase Cß (PLCβ) is a downstream effector of activated $G\alpha_q$. It hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) into diacyl glycerol (DAG) and inositol triphosphate (IP3) (Makhlouf and Murthy, 1997; Singer et al., 1997). These downstream effectors can directly activate synaptic transmission or muscle contraction (Zhou et al., 1994; Hilfiker and Augustine, 1999; Yawo, 1999; An et al., 2002; Hubbard and Hepler, 2006). In *C. elegans*, PLCβ is encoded by the *egl-8* gene (Lackner et al., 1999). Together with *itr-1,* which encodes the IP3 receptor, *egl-8* was demonstrated to be required for LEV-induced spicule protraction (Gower et al., 2005). To determine if PLCβ transduces the GAR-3(mAChR)/$G\alpha_q$ signal in response to 50 mM oxo M stimulation, I used *egl-8(n488)* (Miller et al., 1999), an allele of PLCβ that deletes exons 10 and 11 and disrupts the reading frame before Y catalytic domain. I found that compared to 86% of wild-type males (n=72), 65% of *egl-8(lf)* males protracted their spicules in the drug (Table 2; n=40, p=0.015). This result suggests that GAR-3(mAChR) promotes spicule protraction partially via activating the PLCβ signaling. In addition, the *egl-8(lf)* mutant males were also resistant to LEV. In 2μM LEV (EC_{90}), only 42% of the mutant males protracted their spicules (n=50), compared to 88% of the wild type (n=40). This indicates that PLCβ, as a downstream effector of the GAR-3(mAChR)/$G\alpha_q$ pathway, is required for wild-type LEV dose response.

However, greater than half of *egl-8(lf)* males responded to oxo M, as compared to 7% of *egl-30(lf)* males with reduced $G\alpha_q$ activity. Therefore, GAR-3(mAChR)/$G\alpha_q$

signaling promotes spicule protraction through other pathways in addition to IP3-DAG signaling. This is not surprising since other $G\alpha_q$- regulated behaviors in *C. elegans*, such as locomotion and egg-laying behavior, have also been suggested to be regulated by a putative EGL-8(PLCβ)-independent pathway {Charlier, 2006 #43;Bastiani, 2003 #207;Miller, 1999 #189;Charlier, 2006 #43}. Recently, trio, which contains a Rho-specific guanine-nucleotide exchange factor (GEF) domain, has been suggested to be a downstream effector of $G\alpha_q$ (Rojas et al., 2007). It was found that in parallel with PLCβ, trio also regulates locomotion in *C. elegans* (Williams et al., 2007). To determine whether trio is also a downstream effector of the GAR-3(mAChR)/$G\alpha_q$ signaling in regulating spicule activity, I asked if trio loss-of-function mutant males also have reduced oxo M sensitivity. Trio is encoded by the *unc-73* gene, and *unc-73(ce362)* is a loss-of-function allele in which the Rho-GEF domain is disrupted (Williams et al., 2007). In 50mM oxo M (EC_{90}), 37% of the *unc-73(lf)* males protracted their spicules (Table 2; n=35, $p<0.0001$). I then made the *unc-73(lf);egl-8(lf)* double mutant, and found only 12% of the males responded to 50 mM oxo M (Table 2; n=75, $p<0.0001$). This suggests that the GAR-3(mAChR)/$G\alpha_q$ signaling promotes spicule protraction via both phospholipase Cβ and Trio Rho-GEF. Nevertheless, given Rho is a small GTPase that has an important role in regulating cell cytoskeleton organization as well as other cellular processes (Bishop and Hall, 2000), the possibility that trio Rho-GEF is essential for normal cell physiology which is required for oxo M-induced spicule protraction has not been ruled out.

GAR-3(mAChR) is expressed in the circuit that regulates male spicule insertion

To determine where GAR-3(mAChR)/Gα_q signaling functions to regulate spicule protraction, I checked the expression pattern of the *gar-3* gene. Previous study by Stager and Avery (2004) has reported that *gar-3* is expressed in pharyngeal muscles and the I3 neuron, along with other extrapharyngeal neurons. They determined this by using 6 kb of DNA sequence upstream of the *gar-3* start codon as promoter to drive GFP expression (Steger and Avery, 2004). When I used the same 6 kb sequence (I referred it as the P*gar-3A* promoter) to drive YFP expression (Fig. 6), in addition to what has been reported before, I found it was expressed in the body wall muscles, the male diagonal muscles, and one of the neurons in the male ray 8. However, no expression was found in cells that are closely related to the spicule protraction behavior.

According to cDNA expression tag data listed on www.wormbase.org, there is an alternative 5'UTR located ~10 kb upstream of the *gar-3* start codon (Hwang et al., 1999)(http://www.wormbase.org; stable release WS160). It is possible that sequence upstream to this UTR can also be used as promoter region to drive *gar-3* expression. To test this, I fused 3.5 kb of sequence (P*gar-3B*) upstream of the *gar-3* isoform Y40H4A.1a.a to the YFP gene (Fig. 6). I found that this construct is expressed in the SPC sensory-motor neurons, the PCA and PCB p.c.s. neurons, the male spicule protractor muscles, the anal depressor muscle, the VD and DD ventral cord neurons, some tail and nerve ring neurons, and the body wall muscles (Fig. 3*C*, *D*). Among these cells, the SPC, PCA and PCB neurons and the spicule protractor muscles have been shown to regulate spicule protraction behavior (Garcia et al., 2001). In hermaphrodite,

this construct is expressed in the same set of cells, except for those specific to males (Fig. A-4).

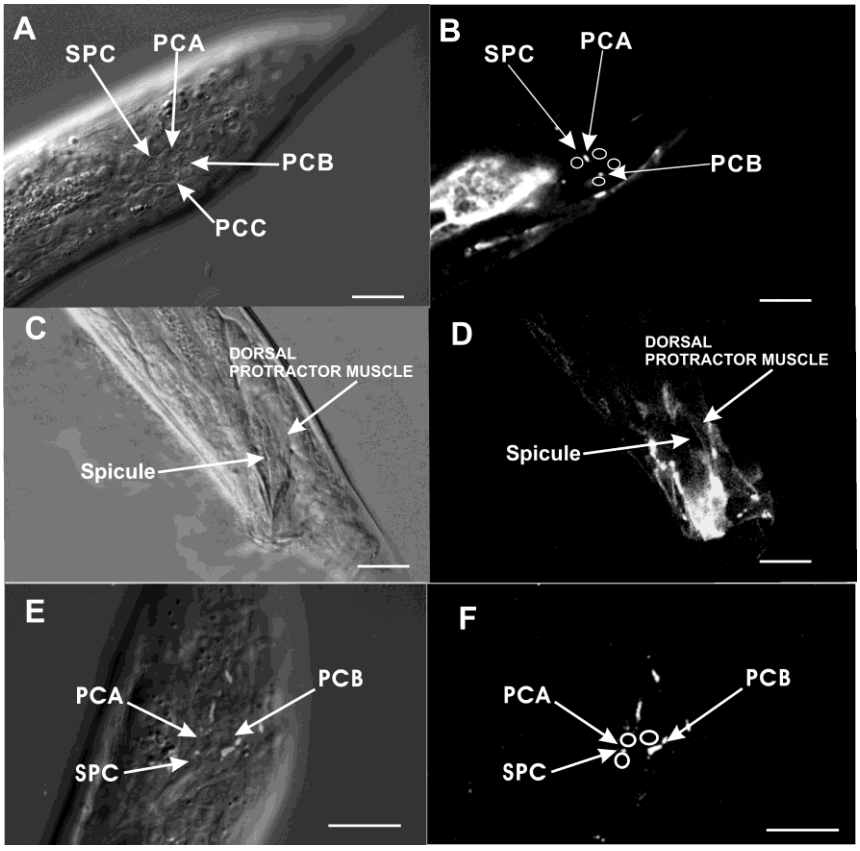

Figure 7. Native and heterologous expression of the *gar-3*::*YFP* translational fusion protein.
A. DIC and ***B.*** fluorescence images of the left lateral tail region of a mid L4 male expressing both *Pgar-3A:gar-3::YFP* and *Pgar-3B:gar-3::YFP*. Nuclei of the neurons are labeled with arrows. In panel ***B,*** positions of these neurons are indicated with circles, and localizations of GAR-3::YFP on the cell membranes are denoted by arrows.
C. DIC and ***D.*** fluorescence images of left lateral tail region of an adult male expressing *Punc-103E:gar-3::YFP*.
E. Merged DIC and fluorescence and ***F.*** fluorescence images of left lateral tail region of a mid L4 male expressing the *Punc-103F:gar-3::YFP*. In panel ***E.*** nuclei of neurons are labeled with arrows. GAR-3::YFP localization on cell membranes can be seen as white puncta. In panel ***F,*** neuronal positions are indicated with circles, and localizations of GAR-3::YFP on the cell membranes are denoted by arrows. Scale bar is 10 μm.

GAR-3 on both neurons and muscles facilitates spicule protraction

To determine which cells GAR-3(mAChR) functions in to regulate the spicule protraction behavior, I fused YFP to the last codon of the *gar-3* gene, and used different promoters to drive its expression. Using YFP, the fusion protein expression could be visualized and was found on the membranes and neuron processes (Fig. 7*A*, *B*). The oxo M sensitivity of the *gar-3(lf)* males could only be restored when I used the P*gar-3B* to drive the fusion protein expression, but not P*gar-3A* (Table 2). This supported that GAR-3(mAChR) functions in the SPC, PCA, and PCB neurons and the spicule protractor muscle to regulate spicule activity.

The cholinergic SPC neurons directly synapse the spicule protractor muscles, whereas it was proposed previously that the p.c.s. neurons PCA and PCB might control protractor contraction through indirect connection (for example, through chemical or electrical connections to the SPC). To further determine whether GAR-3(mAChR)/$G\alpha_q$ signaling functions in the presynaptic cells or the postsynaptic cells to promote spicule protraction, I expressed the *gar-3::YFP* fusion protein under control of the P*unc-103E* and P*unc-103F* promoters (Gruninger et al., 2006; Reiner et al., 2006). The P*unc-103E* promoter expresses in the protractor muscles but not in any neurons that innervate them (Fig. 7*C*, *D*). The P*unc-103F* promoter expresses in the SPC, PCA, and PCB neurons (Fig. 7*E*, *F*). I found the *gar-3:YFP* fusion protein expressed by either promoter could restore *gar-3(lf)* males' ability to protract their spicules in 50 mM oxo M (Table 2). 92% of the *gar-3(lf)* males that expressed *gar-3* in sex muscles protracted spicules (n=13),

and 97% of the ones that expressed *gar-3* in spicule-associated neurons did it (n=29). In addition, laser-ablating the SPC and the PCB neurons restored oxo M resistance to the *gar-3(lf)* males that expressed P*unc-103F:gar-3::YFP* (none of the males protracted spicules in 50 mM oxo M, n=7, $p<0.0001$), but not to the males that expressed P*unc-103E:gar-3::YFP* (62.5% of males protracted spicules in 50 mM oxo M, n=8, $p>0.05$). These suggest that GAR-3(mAChR) that expressed in both presynaptic cells and postsynaptic cells contributes to promote spicule protraction. The oxo M sensitivity was fully restored using either muscle-expressed GAR-3 or neuron-expressed GAR-3, possibly because both constructs were over-expressed.

Similarly, overexpressing the *gar-3* gene in either male sex muscles or the spicule-related neurons using the P*unc-103E* and P*unc-103F* promoter, respectively, restored the *gar-3(0)* mutant males' LEV sensitivity. In 1μM LEV, in contrast to the *gar-3(0)* mutant males, 82% of the males that expressed *gar-3* in sex muscles protracted their spicules (n=28, $p<0.05$), and so did 75% of the males that had *gar-3* overexpressed in the spicule-related neurons (n=24, $p<0.05$). This, again, suggests that both presynaptic and postsynaptic GAR-3(mAChR), when overexpressed, facilitate the spicule circuit activity.

In *gar-3(lf)* males, the ability of muscle-expressed GAR-3(mAChR) to rescue oxo M sensitivity suggested that the drug can by-pass the presynaptic neurons and directly induce muscle contraction. However, this contradicts the earlier result that cholinergic synaptic transmission was required for oxo M-induced spicule protraction, since the *unc-64(lf)* and *unc-17(lf)* mutant males had reduced oxo M sensitivity. I

reasoned that although over-expressed GAR-3(mAChR) on the protractors can by-pass the neuron function, endogenous level of GAR-3 receptors on the muscles might not be sufficient to mediate oxo M-promoted behavior. To test this hypothesis, I ablated the *gar-3*-expressing cholinergic SPC and PCB neurons, either individually or in combination, in the wild-type males, and asked if loss of these neurons impairs sensitivity to oxo M. 11% of the SPC-ablated males (n=19, $p<0.0001$), 30% of PCB-ablated males (n=20, $p<0.0001$) and 28% of double-ablated males (n=67, $p<0.0001$ vs. intact males; $p=0.14$ vs. SPC-ablated males) responded to the agonist with spicule protraction (Table 2). This data suggests that in the absence of SPC and PCB, endogenous levels of muscular GAR-3(mAChR) are not sufficient to mediate oxo M-induced spicule protraction. In addition, ablation of SPC and PCB neurons in *gar-3(lf)* males that had GAR-3(mAChR) over-expressed on the protractors still resulted in ~88% (n=8) of transgenic males protracting their spicules in the drug, confirming that when over-expressed, muscular GAR-3 is sufficient to promote spicule protraction.

Loss of GAR-3 function results in males that have defect in penetrating the vulva during mating

My molecular, mutational and pharmacological studies suggested that a GAR-3(mAChR)/$G\alpha_q$ pathway functions on spicule-related neurons and muscles to enhance the behavioral output of the ionotropic AChRs signaling and promote spicule protraction. However, I did not know how this signaling pathway was used by the male

to regulate his behavior. To answer this question, I asked if the *gar-3(lf)* mutant males had any behavioral defect during mating. I found that these males could execute every steps of mating without obvious behavioral defect (data not shown). I reasoned that, like a lot of $G\alpha_q$-mediated signaling, the GAR-3(mAChR) signaling might play a modulatory role in regulating behavior, thus the male behavior needed to be examined more carefully.

The *gar-3* gene is expressed in two of the p.c.s. neurons, which were suggested to regulate the rhythmic shallow spicule thrusts at the vulva (Garcia et al., 2001). To address if GAR-3 functions in these neurons to modulate this behavior, I asked if *gar-3(lf)* males had defective spicule prodding behavior. Mutant males that lacked a ryanodine Ca^{2+} channel have been shown to have impaired spicule prodding behavior, and that the frequency of repetitive thrusts of these males was much lower than the wild type (Garcia et al., 2001). However, the *gar-3(lf)* males display similar frequency of thrusts (6.2 +/-compared 1.2 contractions/sec) compared to wild type (5.8 +/- 0.7 contractions/sec; p=0.6), suggesting the spicule circuit does not require the GAR-3(mAChR) signaling to regulate the muscle contraction-relaxation cycle.

The vulva of a young adult hermaphrodite (~18hr after molt) is difficult for males to breach, so the males need to sustain their tail position at the vulva in order to repetitively attempt to insert spicules (Garcia et al., 2001). I found that if the hermaphrodite vulva was not breached instantaneously, the *gar-3(lf)* males moved off the vulva rather than sustaining their attempts to penetrate the vulva. In a 10 min assay, I counted the number of times a male made contact with the hermaphrodite's vulva using

his tail until he completely inserted spicules or the 10 minutes ran out. I found that wild-type males moved on and off the vulva on average 6.3 times (n=26), whereas the *gar-3(lf)* males (n=29) moved on and off the vulva on average 17.5 times ($p<0.001$) during their spicule insertion attempts (Fig. 8). This phenotype could be a result of the males' defect in inserting their spicules efficiently, or defect in sustaining vulva contact, or due to a combination of both. This suggests that when the task of breaching the vulva is prolonged, GAR-3(mAChR) is required for the integrated function of the postcloacal sensilla neurons, the SPC neurons and the protractor muscles, which facilitates spicule insertion attempts and the vulva location behavior.

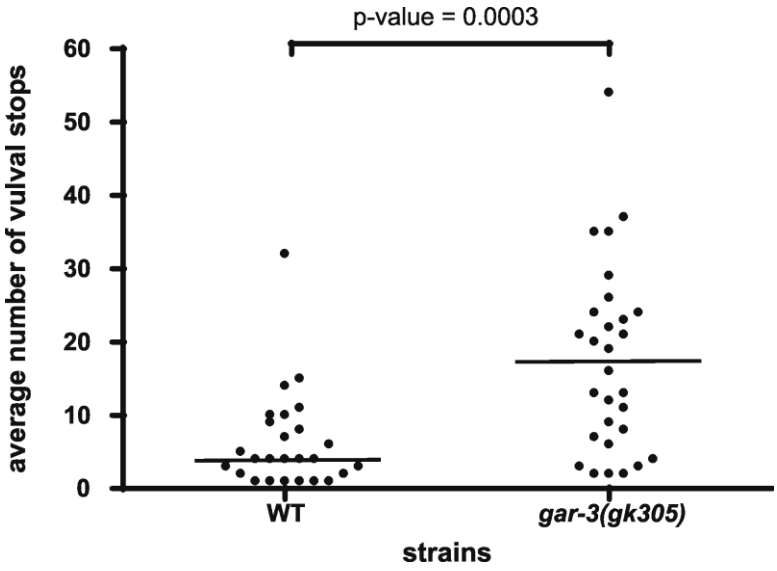

Figure 8. The *gar-3(lf)* males employ more spicule insertion attempts to achieve vulva penetration. Spots represent the total number of vulval stops (insertion attempts) employed by individual males. 26 wild-type males and 29 *gar-3(lf)* males were assayed. The horizontal bar indicates the sample median. The p-value was calculated using the Mann-Whitney test.

Chapter summary

In Chapter III, I demonstrated a GAR-3(mAChR)-mediated signaling pathway that is coupled to $G\alpha_q$ to facilitate the insertion of *C. elegans* male copulatory spicules into the hermaphrodite's vulva. When treated with a GAR-3-specific mAChR agonist, oxo M, wild-type males protracted their spicules. Using this drug-induced behavior as readout, I have been able to identify molecules that are involved in this signaling pathway by testing mutant males' responses to oxo M. I found that PLCβ and trio Rho-GEF are likely to function downstream of $G\alpha_q$ in the spicule circuit. Both of them have been reported to regulate synaptic transmission (Steven et al., 2005; Williams et al., 2007). Consistent with that, expressing GAR-3 in neurons that regulate spicule activities restored oxo M sensitivity in the *gar-3(lf)* mutant males. This suggests that the GAR-3(mAChR)/$G\alpha_q$ signaling upregulates synaptic transmission in the male spicule circuit. As the muscular ionotropic AChRs are also required for oxo M-induced spicule protraction, it is likely that increased cholinergic synaptic transmission promotes spicule protraction via activating ionotropic AChRs on the protractor muscles.

The role of endogenous GAR-3(mAChR) is probably to facilitate spicule insertion during mating, as the *gar-3(lf)* mutant males could not insert their spicules as efficiently as the wild type, when paired with hermaphrodites. I propose that the GAR-3(mAChR)/$G\alpha_q$ signaling is likely to promote spicule protraction by enhancing the behavioral output of the postsynaptic ionotropic AChRs signaling in the protractor muscles. A previous study has suggested that spicule protraction is a behavioral output of endogenous ACh activating ionotropic AChRs on the spicule protractor muscles. It

was also shown that exogenous ACh agonists (nicotine, arecoline and LEV) could mimic the effect of ACh to induce spicule protraction. Here I found that both GAR-3(mAChR) and $G\alpha_q$ were required for postsynaptic ionotropic AChR-mediated spicule protraction, and so was the cholinergic synaptic transmission between the spicule-related neurons and the protractor muscles. This indicates a scenario that before or during mating, the presynaptic neuron function, which is regulated by the GAR-3(mAChR)/$G\alpha_q$ signaling, facilitates spicule insertion by enhancing the postsynaptic ionotropic AChRs motor output.

CHAPTER IV

CHOLINERGIC NEUROTRANSMISSION FACILITATES MALE.S CONTACT WITH HERMAPHRODITE DURING COPULATION

Males utilize ionotropic AChRs to maintain contact with the hermaphrodite vulva during mating

When a *C. elegans* male initiates contact with a hermaphrodite as an attempt to mate, the hermaphrodite moves away. For the sperm-carrying hermaphrodites, mating is not required for reproduction. Thus, an essential step for the male to successfully mate is to sustain precise contact between his cloaca opening and the hermaphrodite's vulva so that he has enough time to insert his spicules.

In the last chapter, I showed that mutant males, lacking of a muscarinic acetylcholine receptor (mAChR) GAR-3, are less efficient in inserting their spicules into the hermaphrodite's vulva during mating, compared to the wild type. The mutant males need to take more vulval encounters before they can penetrate the vulva slit with their spicules (Fig. 8). This defect could be a result of either the male being less proficient in maintaining contact between his cloaca and the hermaphrodite's vulva, or delayed spicule insertion, or both. This mAChR is expressed in the cholinergic spicule-related neurons, PCB and SPC, to enhance ACh secretion from these neurons (Liu et al., 2007). ACh secreted from the SPC neurons activates ionotropic ACh receptors on the spicule

protractor muscles to induce muscle contraction, which results in tonic spicule protraction (Garcia et al., 2001). It has been shown that the LEV-sensitive ACh receptor (L-AChR), which contains three α subunits, UNC-38, UNC-63, and LEV-8, and two non-α subunits, UNC-29 and LEV-1, is required for the ACh agonist-induced spicule protraction (Ballivet et al., 1996; Fleming et al., 1997; Richmond and Jorgensen, 1999; Garcia et al., 2001; Rayes et al., 2007). I speculated that the ionotropic ACh receptors in the cells of the spicule circuit can also be used to sustain precise vulval contact behavior; therefore in the *gar-3* deletion mutant, reduced cholinergic synaptic transmission between the spicule-related neurons and the sex muscles resulted in defective vulval contact.

To address whether ionotropic AChRs are required for other steps of male mating, I asked how well mutant males that lack functional L-AChR can mate, compared to the wild type. I used mutant males containing the *unc-29(e193)* allele, which has a missense mutation that changes a proline into serine at amino acid 258, within the hypothetic first transmembrane domain of the UNC-29 non-α subunit (Lewis et al., 1980a; Lewis et al., 1980b; Kim et al., 2001). To verify if the *unc-29(e193)* mutant males are deficient for the L-AChR in the spicule protraction circuit, I asked if the mutant males protract their spicules in response to LEV. The effective concentration of LEV that can induce prolonged protraction in 90% of the males (EC_{90}) is 2μM (Garcia et al., 2001). I found that at this concentration, none of the mutant males protracted their spicules (n=47), compared to 88% of the wild type (n=57, $p<0.0001$), suggesting that there is no functional L-AChR in the spicule circuit. However, as *unc-29* is also

expressed in the body wall muscles to regulate locomotion, the *unc-29(e193)* allele causes worms to have severe uncoordinated locomotion (Richmond and Jorgensen, 1999). Therefore, *unc-29(lf)* males are challenged to move backward along the hermaphrodite during mating. I then used the upstream region of the *acr-8* gene to express *unc-29* cDNA (P*acr-8:unc-29*cDNA::SL2::*GFP*) in the body wall muscles but not in the spicule-related sex muscles in the mutant males (LeBoeuf et al., 2007). This restored the wild-type locomotion to these males, but it still left the spicule circuit deficient for the L-AChR function. Indeed, compared to the *unc-29(lf)* mutant, the transgenic males were still resistant to LEV; 6% of them protracted spicules in 2μM LEV (n=35, p=0.18). To assess the mating potency of the locomotion-restored *unc-29(lf)* males, I paired each male with a free-moving hermaphrodite on a small bacterial food-lawn for four hours. I found that the transgenic males can sire progeny with 83% of the wild-type efficiency (Fig. 9; n=38, p>0.05). This indicates that although the L-AChR is used for ACh-agonist-induced spicule protraction, other receptors might compensate for its function in *unc-29(lf)* males during mating.

Next, I asked which other ionotropic AChRs could be functioning in the male spicule circuit. It has been reported that the homomeric nAChR formed by the α7-like nAChR subunits ACR-16 function in parallel with the L-AChR in the *C. elegans* locomotion circuit (Ballivet et al., 1996; Touroutine et al., 2005). Thus, I asked if ACR-16 is also functioning during the male mating behavior. In the 4-hour mating potency assay, males that contain the *acr-16(ok789)* deletion allele could achieve 88% of wild-type mating potency (Fig. 9; n= 41, p>0.05), suggesting that like the L-AChR, the ACR-

16 nAChR is not essential for the male potency. I reasoned that if the L-AChR and the ACR-16 containing nAChR are required for mating, but they are interchangeable, then a severe mating defect can only be seen in the *unc-29(e193);acr-16(ok789)* double mutant. The double mutant had more severe locomotion defect than the *unc-29(lf)* worms, but could be rescued by restoring *unc-29* in the body wall muscles using the construct of P*acr-8:unc-29*cDNA::SL2::*GFP*, since the *acr-16(lf)* worms have wild-type locomotion (Ballivet et al., 1996; Touroutine et al., 2005). As I predicted, the *unc-29(lf);acr-16(lf)* males could only achieve 15% of wild-type mating potency in the 4-hour assay (Fig. 9; $n=33$, $p<0.05$). I then concluded that both the L-AChR and the ACR-16 ionotropic AChR are used in the male mating behavior, and when one receptor is non-functional, the other can compensate.

To understand which aspect of mating requires the function of the L-AChR and the ACR-16-containning nAChR, I observed mating behavior of the locomotion-restored double mutant males. I paired the males with genetically paralyzed *unc-64(lf)* hermaphrodites, so each step of mating could be easily video recorded and accurately analyzed. During a 5 minute observation window, the majority of the males I assayed made multiple contacts with the hermaphrodites' vulva before they inserted their spicules or until the observation period ended. I noticed that once the transgenic double mutant males located vulva with their tails, they fell off the vulva more frequently than the wild type. When I measured the average vulval contact duration for these males, they could maintain the contact for half the time of wild-type males (Fig. 9; $p=0.004$). I further asked if the defect in sustaining vulval contact was due to loss of functional L-

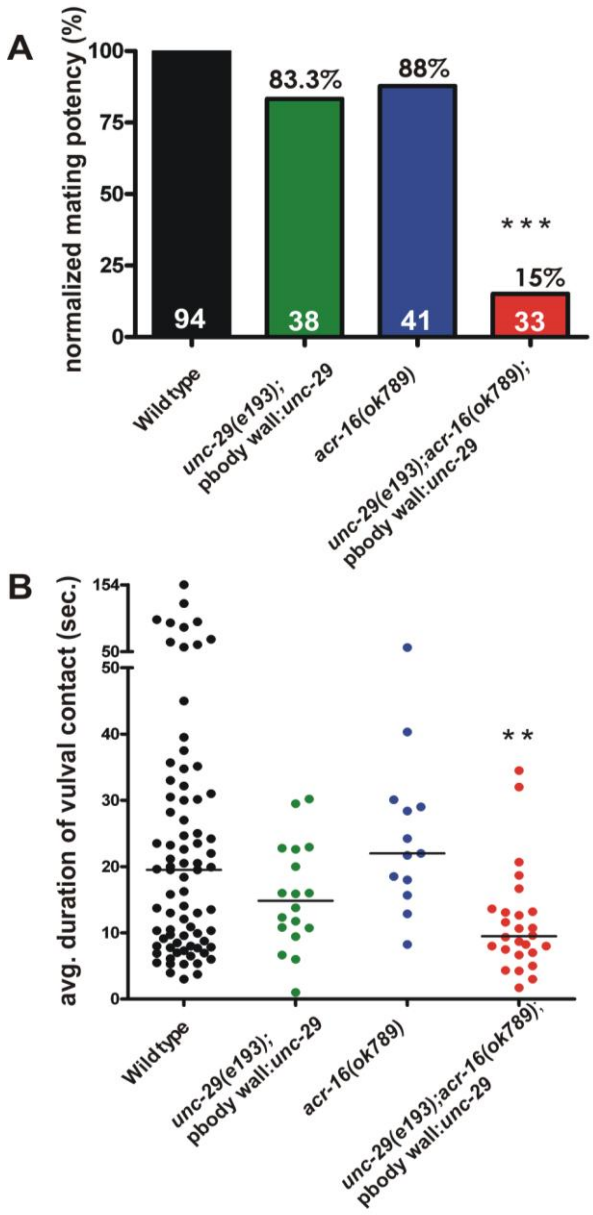

Figure 9. Ionotropic AChRs-mediated synaptic transmission is required for prolonged vulva contact.

A. Relative mating potency of mutant males normalized to wild type. The numbers of males assayed for each strain are listed within the bars. The number for wild type is an accumulated number. Asterisks (***) indicate the *p* value<0.0001, calculated using the Fisher's exact test.

B. Profiles of average duration of vulva contact. The spots represent the average duration of vulva contact of individual males. The horizontal bar indicates the sample median. Asterisks (**) indicate the *p* value<0.005, calculated using the Mann-Whiney test.

AChR, the ACR-16 nAChR or both. Neither the *unc-29(e193)* mutant males nor the *acr-16(ok789)* mutant males had obvious vulval contact defect (Fig. 9), suggesting that these genes are used redundantly to maintain vulval contact.

Interestingly, the vulval contact defect of the *unc-29(lf);acr-16(lf)* double mutant did not seem to severely affect mating potency of the mutant males when they mated with the paralyzed hermaphrodites, since eventually 48.5% of mutant males inserted their spicules into the hermaphrodite's vulva during mating (n=33), which was not statistically different from the 69.7% of the wild type (n=33, $p>0.05$). However, when mated with free-moving hermaphrodites, this difference is likely to be amplified by the fact that the hermaphrodites can escape, so if a male loses contact with the vulva it is difficult for him to locate the vulva again. Consistent with this idea, only 10% of the wild-type males could impregnate the free-moving hermaphrodites within 10 minutes (n=20), as maintaining contact with a moving object is challenging for wild-type males.

The *unc-29* and *acr-16* genes are expressed in the male-specific muscles

To determine where the L-AChR and the ACR-16-containing AChR function in the male to facilitate the vulval contact behavior, I asked where these receptors are expressed in the male tail. I injected a YFP expression construct that contains 5.3 kb DNA sequence upstream of the *acr-16* first ATG. In adult males, this construct was expressed in the body wall muscles and some male-specific muscles, including the anal depressor, the spicule protractors, the gubernacular erector and retractor muscles, and the

anterior oblique muscles (Fig. 10*A-D*). However, it was not seen in any of the neurons that regulate the spicule activities.

The *unc-29* gene encodes a L-AChR non-α subunit. Formation of a functional channel requires all five subunits: the α subunits encoded by *unc-38*, *unc-63* and *lev-8*, and the non-α subunits encoded by *unc-29* and *lev-1* (Ballivet et al., 1996; Fleming et al., 1997; Richmond and Jorgensen, 1999; Garcia et al., 2001; Rayes et al., 2007). In Chapter III, I examined the expression pattern of the *unc-38* gene by expressing YFP driven by the 1.1 kb sequence upstream of the *unc-38* first ATG. It was expressed in the male spicule protractor muscles and the body wall muscles, but not in any spicule-related neurons. To verify this result, I fused a 2.2 kb sequence, which includes 1.3 kb sequence upstream of the *unc-29* start codon and the genomic sequence of the gene up to the second intron, to the YFP gene (P*unc-29:YFP*). Similar to the *unc-38* expression construct, this construct expressed the YFP in the spicule protractors, the anal depressor muscle and the body wall muscles. It was also expressed in some ventral cord neurons and head neurons (Fig. A-1).

Nonetheless, the non-coding regions inside of a gene can sometimes also be important to determine the gene's expression pattern (Blanchette and Tompa, 2002; Okkema and Krause, 2005). I asked if this could be the case for *unc-29*. The 1.3 kb sequence upstream of the first exon of *unc-29*, along with the whole *unc-29* genomic sequence, was fused to the YFP gene. I found that adding the genomic sequence extended the expression pattern to the male-specific gubernacular erector and retractor muscles and the anterior oblique muscles, but still not any spicule-related neurons (Fig.

10*E-H*). Therefore, *acr-16* and *unc-29* are both expressed on the male-specific muscles in the male tail.

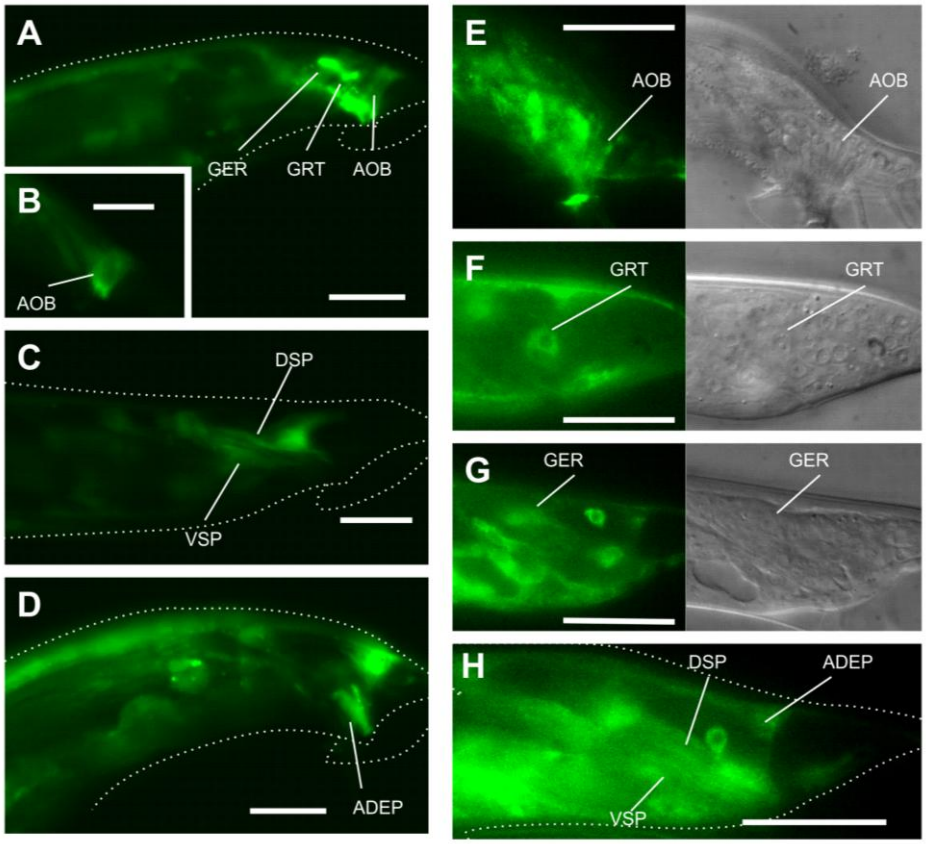

Figure 10. Male tail expression of *acr-16* and *unc-29*.
A-D. Fluorescence images of the tail region of adult males expressing the P*acr-16:YFP* construct. Expression can be seen in the gubernacular erector (GER), gubernacular retractor (GRT), anterior oblique (AOB), dorsal spicule protractor (DSP), ventral spicule protractor (VSP), and anal depressor (ADEP) muscles. Scale bar, 20µm.
E-H. Fluorescence images of the tail region of adult males expressing the P*unc-29:unc-29::YFP* construct. Expression can be seen in the gubernacular erector (GER), gubernacular retractor (GRT), anterior oblique (AOB), dorsal spicule protractor (DSP), ventral spicule protractor (VSP), and anal depressor (ADEP) muscles. Scale bar, 20µm. The dotted lines depict the outline of the mail tail.

I also asked if *unc-38* is also expressed in these muscles to form functional receptor with *unc-29*. Using an *unc-38* promoter-genomic DNA fusion construct to drive YFP expression, I found that it was expressed in every male-specific muscle (including the anal depressor, the spicule protractors and retractors, the diagonal muscles, the gubernacular muscles, the oblique muscles and the sphincter), and surprisingly, the SPC neurons (Fig. A-1). Similarly, another L-AChR α subunit-encoding gene, *unc-63*, was also found expressed in these cells. The 2.4 kb sequence upstream of *unc-63* start codon, including the first three codons, was fused to the YFP gene. This construct was expressed in every male-specific muscle and also in the SPC neurons (Fig. A-1).

The expression patterns of *unc-38* and *unc-63* indicate that these α subunits can form a nAChR on the SPC neurons independent of *unc-29*. Indeed, a recent study has reported a LEV-insensitive nAChR in *C. elegans* ventral cord cholinergic motor neurons (Jospin et al., 2009). This receptor is formed by the UNC-38, UNC-63 and ACR-12 α subunits and the ACR-2 and ACR-3 non-α subunits. Consistent with this, the *acr-12* promoter-genomic DNA fusion construct is also expressed in the SPC neurons, indicating that these neurons have the functional LEV-insensitive nAChR (Fig. A-1). Therefore, in this study, by using the *unc-29(lf)* allele, I only disrupted the function of the L-AChR in the male-specific muscles, but not the nAChR on the spicule neurons.

The postcloacal sensilla neurons are required for maintaining the male tail position at the vulva

Both *acr-16* and *unc-29* were determined to express specifically in the male sex muscles, and loss of their functions in these muscles interfered with the male's ability to stay in contact with the hermaphrodite's vulva. I hypothesized that, in the male copulation circuit, certain neuromuscular junctions are important for maintaining vulval contact. The cholinergic synaptic transmission at these neuromuscular junctions is impaired in the *unc-29(lf);acr-16(lf)* double mutant males, so they could not sustain their tail position at the vulva.

To address which neurons in the male tail regulate the vulval contact behavior, I asked what neurons synapse the *acr-16*- and *unc-29*-expressing muscles, by studying the connections of the male-specific nervous system. I noticed that the spicule protractors are innervated by the SPC neurons, and the anterior oblique muscles (left/right), the gubernacular erector (L/R) and retractor (L/R) muscles are innervated by the postcloacal sensilla (p.c.s.) neurons (Fig. 11; S. W. Emmons, personal communication, 2010). I then asked if ablating these neurons would affect the male's ability to stay with the vulva.

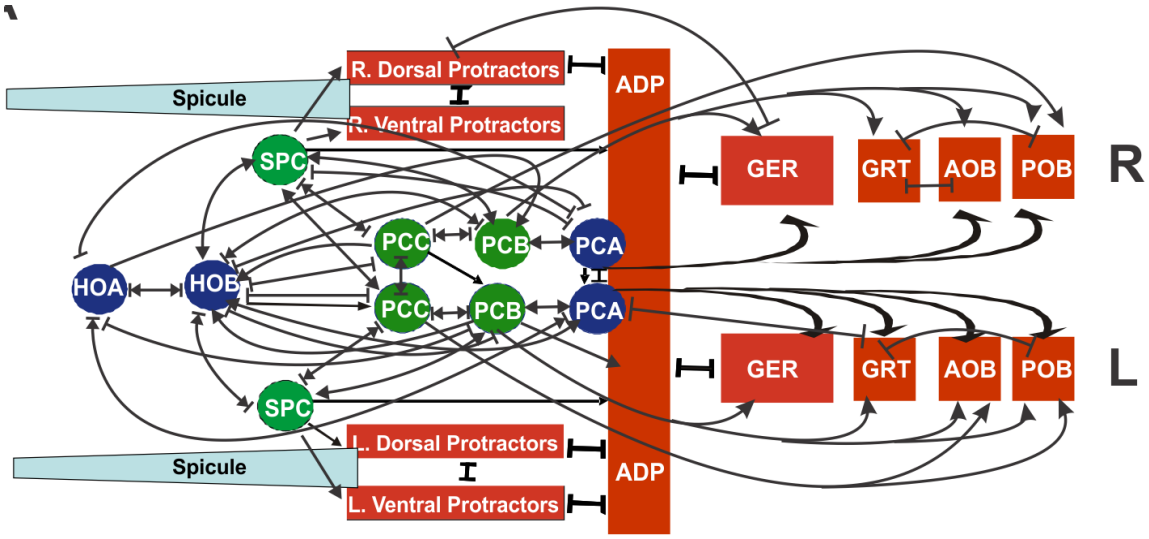

Figure 11. Circuit diagram of cells that control spicule motion. Gubernacular erector (GER), gubernacular retractor (GRT), anterior oblique (AOB), posterior oblique (POB), dorsal spicule protractor (DSP), ventral spicule protractor (VSP) and anal depressor (ADP). Abbreviated cartoon of limited connections between left and right pairs of neurons and muscles discussed in this work [adapted from (Sulston et al., 1980), and the Male Wiring Project]. Refer to (Male Wiring Project, Albert Einstein College of Medicine, http://worms.aecom.yu.edu/pages/male_wiring_project.htm) for a more complete list of connections to other cells in the male. Arrows and bars indicate chemical synapses and gap junctions, respectively. Bi-directional arrows refer to cells that make reciprocal chemical synapses. Bi-directional arrows embedded in bars refer to cells that make gap junctions in addition to reciprocal chemical synapses. The green circles refer to cholinergic neurons.

The p.c.s. neurons have been suggested to sense the precise position of the hermaphrodite's vulva. Males that had one pair of these neurons ablated but the other two pairs intact could still locate the vulva (Liu and Sternberg, 1995). However, males with zero or only one pair of the p.c.s. neurons will generally pass over the vulva without

stopping, even though they can sense the general area of the vulva using the hook sensillum neurons (Liu and Sternberg, 1995; Barr and Sternberg, 1999). Although these neurons have been shown to sense the vulva, it has not been determined if they also function to maintain contact between the male's cloaca and the hermaphrodite's vulva. To address this question, I ablated each individual pair of these neurons in males at the L4 larval stage and observed their mating behavior when they became adults. Consistent with what was reported before, the operated males could locate the vulva without any difficulty. However, when I quantified the average time each operated male could sustain vulval contact, I found that similar to the *unc-29(lf);acr-16(lf)* mutant males, the operated males with any pair of the p.c.s. neurons ablated had reduced duration of vulval contact (Fig. 12*A*). This suggests that each pair of the p.c.s. neurons continues contributing to the male's ability to sustain vulval contact after the initial recognition of vulva. Among the p.c.s. neurons, the PCBs and PCCs are cholinergic. Therefore, it is possible that they synapse the postsynaptic muscles and activate ionotropic AChRs on these muscles to exert their function in mating.

Prolonged spicule protraction has been shown to require the cholinergic SPC neurons. Males that had the SPCs ablated failed to insert their spicules during mating (Sulston et al., 1980; Garcia et al., 2001). However, I found ablation of these neurons did not reduce the duration of vulval contact; instead, the operation enabled the males to stay even longer over the vulva (Fig. 12*B*). This increase in duration is not a result of failure to insert spicules, as majority of the intact control males I observed inserted their spicules after a couple of times of vulval contacts and multiple contacts were recorded

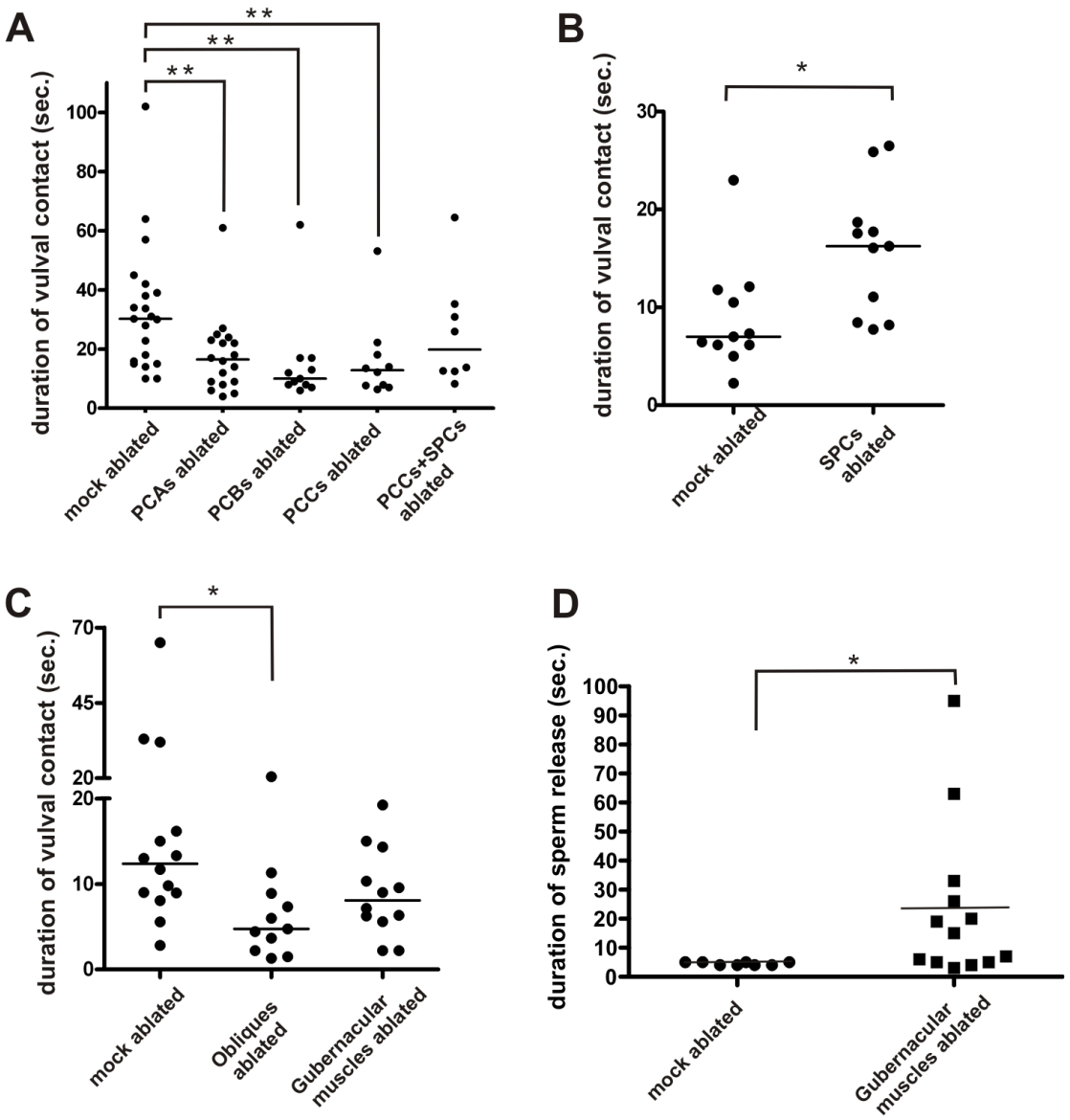

Figure 12. The postcloacal sensilla (p.c.s.) neurons and the oblique muscles facilitate prolonged vulval contact.

A-C. The spots represent the average vulval contact duration for each individual male. The horizontal bar indicates the sample median. Ablated males are compared to control males that mate with the same group of hermaphrodites.

Asterisks (**) indicate the *p* value <0.005, (*) indicates the *p* value <0.05 calculated using the Mann-Whitney non-parametric test.

D. Ablation of gubernacular muscles increases the duration of sperm release. Asterisk (*) indicates the *p* value <0.05 calculated using the Mann-Whitney non-parametric test.

for the operated males as well. In addition, ablating the SPC neurons could reverse the vulval contact deficiency caused by ablation of one pair of the p.c.s. neurons (PCCs) (Fig. 12*A*). Therefore, I reasoned that, though not required for sustaining vulval contact, the SPC neurons might be used to delimit the duration of this step of mating, which eventually could also enhance the efficiency of male mating.

The oblique muscles are used to prolong vulval contact

To address whether the p.c.s. neurons prolong the male vulval contact via activating ionotropic AChRs on the oblique muscles and the gubernacular muscles, I ablated these muscles and explored if the operation affects males' ability to stay in contact with the vulva. The anterior and posterior oblique muscles lie dorsal-ventrally at each side of the male tail, posterior to the spicules. These muscles contain a single sarcomere oriented dorsoventrally, so it was suggested when they contract they generate a downward force to change the posture of the male tail (Lints and Hall, 2009). I ablated these muscles in males at the L4 larval stage and observed their mating behavior with paralyzed hermaphrodites when they became adults. Like the *unc-29(lf);acr-16(lf)* males and p.c.s.-ablated males, these males could not maintain their vulval contact as well as the intact control males (Fig. 12*C*), but they could perform other behavioral steps prior to vulval contact well. The residual vulval contact displayed by the operated males was largely due to the hermaphrodites being paralyzed. When I paired these males with free-moving hermaphrodites, the average duration of vulval contact dropped to 2 seconds

(n=3). This data suggests that the oblique muscles postsynaptic to the p.c.s. neurons are utilized by the copulation circuit to prolong vulval contact. In addition, based on these muscles' position and their shapes in the male tail, I propose that they facilitate the contact via applying force between the male cloaca and the hermaphrodite's vulva.

Ablating the gubernacular muscles, on the other hand, did not affect males' ability to stay in contact with the vulva (Fig. 12*C*). However, strikingly, the gubernacular muscle-ablated males showed difficulty in sperm transfer (Fig. 12*D*). In intact males, after sperms left the seminal vesicle, the gametes were briefly held at the distal end of the vas deferens for two to four seconds, before they were released out of the cloaca (Liu and Sternberg, 1995; Schindelman et al., 2006). However, for the gubernacular muscle-ablated males, the sperm was held in the vas deferens for a much longer period, and sometimes the gametes failed to drain out before the male retracted spicules. The gubernaculum is a cuticle structure that lies posterior to the spicules, and it is thought to help guide the extension of the spicules through the male cloaca. The gubernacular erector and retractor muscles attach the gubernaculum to the body wall (Sulston et al., 1980; Lints and Hall, 2009). I speculate that gubernacular muscle contraction not only moves the gubernaculum, but also lifts the adjacent tissues that are on top of the opening of vas deferens. As a result, the lumen of the distal vas deferens is accessible to the cloacal opening, and sperm get released.

The L-AChR functions on the male sex muscles to facilitate vulval contact

The previous data suggest that the cholinergic p.c.s. neurons innervate the oblique muscles to prolong the contact between the male tail and the hermaphrodite vulva, and it is likely that the *unc-29(lf);acr-16(lf)* double mutant males could not sustain vulval contact due to impaired cholinergic synaptic transmission at the neuromuscular junctions. However, *unc-29* is also expressed in the head neurons and some of the ventral cord neurons, thus it is also possible that the L-AChR on these neurons also contribute to the behavior. To address this, I restored the functional L-AChR to the male-specific muscles and the body wall muscles in the *unc-29(lf);acr-16(lf)* double mutant males, by expressing the *unc-29* cDNA under the 3.5 kb promoter region of the tropomyosin gene *lev-11* (P*lev-11:unc-29cDNA::*SL2*::GFP*)(Gruninger et al., 2006). Compared to the locomotion-restored *unc-29(lf);acr-16(lf)* males that had *unc-29* restored only in the body wall muscles, these males could sustain significantly longer vulval contact (Fig. 13). This suggests that the L-AChR on the male sex muscles is used to facilitate vulval contact.

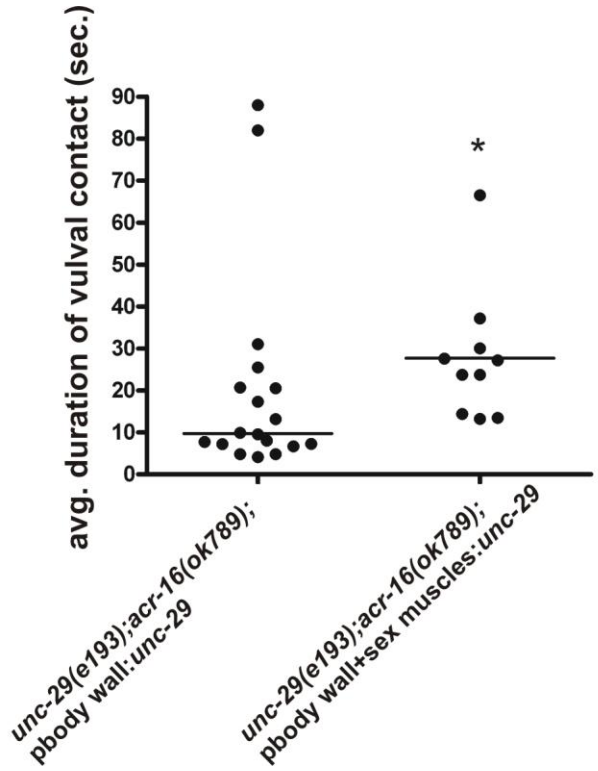

Figure 13. Rescuing *unc-29* in the male sex muscles reverses the vulva contact defect of the *unc-29(e193);acr-16(ok789)* males. The spots represent the average duration of vulva contact of individual males. The horizontal bar indicates the sample median. Asterisk (*) indicates the *p* value<0.05, calculated using the Mann-Whitney test.

Chapter summary

In this chapter, I addressed the neuromuscular circuit used for the male to sustain precise contact between his cloaca and the hermaphrodite's vulva during mating. I noticed that the *unc-29(lf);acr-16(lf)* double mutant males, which did not have the functional L-AChR and the ACR-16-containing nAChR, could not maintain their tail position at the vulva as well as the wild type. I hypothesized that reducing the functions of these ionotropic AChRs disrupts the synaptic transmission between certain cellular

components of the copulation circuit, and these cellular components are used to promote vulval contact. To narrow down the possible postsynaptic cells, I examined the expression patterns of *unc-29* and *acr-16* genes. However, using the upstream sequence of *unc-29* as a promoter to drive YFP expression, did not give me the complete expression pattern of this gene. Instead, I fused both the upstream sequence of *unc-29* and its genomic sequence to the YFP gene. I found it was expressed in male-specific muscles that are used for prolonged spicule protraction, such as the spicule protractors and the anal depressor muscle, and it was also expressed in other male tail muscles, such as the oblique muscles and the gubernacular muscles, whose functions had not been studied before.

The chemical and electrical connections between any two excitable cells in the male tail have been revealed by the Male Wiring Project. By studying these connections, I noticed that the spicule protractors are innervated by the SPC cholinergic neurons, and the oblique muscles and the gubernacular muscles are synapsed by the p.c.s. neurons. I then asked if any of these neurons are used in maintaining vulval contact. By quantifying the contact durations of the males who were ablated with these neurons, I determined that each individual pair of the p.c.s. neurons was required for the wild-type vulval contact behavior, whereas the SPC neurons was not. I then further determined the oblique muscles that are postsynaptic to the p.c.s. neurons are required for vulval contact, but not the gubernacular muscles. The data suggests a model where the cholinergic p.c.s. neurons, after they sense the vulva, release ACh to activate the ionotropic AChRs on the oblique muscles, and contraction of the oblique muscles is

likely to apply a force between the male tail and the hermaphrodite cuticle, in order to cease the backward searching of the male. This model was further supported by the fact that, when I restored functional L-AChR to the male-specific muscles in the *unc-29(lf);acr-16(lf)* double mutant males, it reversed their deficiency in maintaining vulval contact.

CHAPTER V

THE BISTABLE STATE OF THE SPICULE MUSCLE CONTRACTION IS REGULATED BY THE NEURONAL INPUTS OF THE COPULATION CIRCUIT

The gubernacular-oblique muscle group activity directly induces repetitive protractor muscle contractions

In Chapter IV, I showed that for a *C. elegans* male to maintain his tail position at the hermaphrodite's vulva, the p.c.s. neurons need to sense the vulva, and secrete neurotransmitter ACh to activate ionotropic AChRs (L-AChR and ACR-16 nAChR) on the postsynaptic oblique muscles. Activation of ionotropic AChRs causes these muscles to contract and the contraction applies a force against the hermaphrodite cuticle, which keeps the male at the desired position. Interestingly, it was reported in a previous study, and also observed by myself and others, that once a male locats the vulva, his spicules start to rhythmically prod at the vulva; whereas when he loses the vulval contact, the spicule thrusts immediately stops (Garcia et al., 2001). These two motor outputs of the male nervous system, vulval contact and rhythmic shallow spicule thrusts, are correlated with each other. This implied that the two behavioral steps are under regulation of a common mechanism.

It was showed in a previous study that the rhythmic spicule prodding behavior at the vulva is a result of repetitive spicule protractor contraction, and it is triggered by the

p.c.s. neuron activity (Sulston et al., 1980; Garcia et al., 2001). However, what remains unclear is how the p.c.s. neurons signal the protractor muscles, since these neurons have no chemical or electrical connection to the protractors to activate them directly (Sulston et al., 1980)(Male Wiring Project).

To answer the question 'how does information from the p.c.s. neurons relayed to the protractors to cause spicule thrusts', I studied the male circuit connections to determine if any neurons or muscles can bridge the physical connection between these cellular components. I found that if only one neuron or one muscle cell is allowed to connect them, there were only a few options. The p.c.s. neurons have chemical synapses and electrical junctions to the SPC neurons, which directly innervate the spicule protractors and the anal depressor, which is a sex-dimorphic muscle connected to the spicule protractor via gap junctions and is considered to have an accessory role in promoting spicule protraction (Garcia et al., 2001; Garcia and Sternberg, 2003). However, in a previous study, laser-ablation of the SPC neurons did not affect the frequency of spicule prodding at the vulva (Garcia et al., 2001), and my data also showed that this operation did not negatively affect the duration of vulval contacts. Therefore, the p.c.s. neurons must command the protractors via other cells.

Gap junction structures exist between the spicule protractors and the anal depressor muscle (Male Wiring Project). Since gap junctions are low-resistance channels that bridge two cells and allow ions to pass directly, the cytoplasm of these cells are connected and cell activities can be synchronized by exchanging electric current (Bennett et al., 1991; Bennett and Zukin, 2004). Similarly, the gubernacular muscles

(retractor) and the oblique muscles (anterior/posterior) are also connected by gap junctions (Fig. 11; Male Wiring Project), and they are innervated by the same group of presynaptic neurons, the p.c.s. neurons. Therefore I refer these muscles as the "gubernacular-oblique muscle group". Interestingly, gap junction structures are found to connect the protractor-anal depressor muscles that promote spicule movements and the gubernacular-oblique muscle group that is innervated by the p.c.s. neurons (Fig. 11). I hypothesized that through these gap junctions, ions or other signal molecules can pass from the gubernacular-oblique muscles to the protractor-anal depressor muscles to induce Ca^{2+} currents. Therefore when the p.c.s. neurons sense the vulva, they activate the gubernacular-oblique muscles and induce rhythmic shallow contractions of the spicule protractor muscles.

To test this hypothesis, I asked if I directly activate the gubernacular-oblique muscles without activating their presynaptic neurons, would this induce rhythmic contractions of the protractors. I also asked if Ca^{2+} currents can be observed immediately in the protractor-anal depressor muscles upon this stimulation, since muscle contractions are coupled to Ca^{2+} level increases in the cytosol. To depolarize the gubernacular-oblique muscle group at any desired time, I expressed a light-gated cation channel, Channelrhodopsin-2 (ChR2), exclusively on these muscles. Originally found in algae, this channel opens when it is illuminated by the blue light and allows cations, including Na^{+}, K^{+}, H^{+} and Ca^{2+}, to pass, therefore causing strong, rapid and sustained (up to 1 minute) membrane depolarization in *C. elegans* cells (Nagel et al., 2003; Nagel et al., 2005). I used the 6.2 kb region upstream of the *acr-18* gene as the promoter to drive

transcription of ChR2::YFP fusion construct in multiple cells including the gubernacular muscles (erector/retractor) and the posterior oblique muscles (Fig. A-1). Photoactivation of the ChR2 requires the presence of all-*trans* retinal (Nagel et al., 2003). When ChR2 was expressed and activated by growing males in the presence of all-*trans* retinal, the blue excitation light caused the spicule protractors to contract repetitively. When a sequence of blue light pulses were applied, rhythmic spicule thrusts were induced correspondingly and coupled to the light stimulation (Fig. 14*A*; Fig. A-5; see Experimental procedures). In contrast, blue light pulses failed to induce any spicule movement in males that express ChR2 but grew in the absence of all-*trans* retinal (Fig. 14*A*).

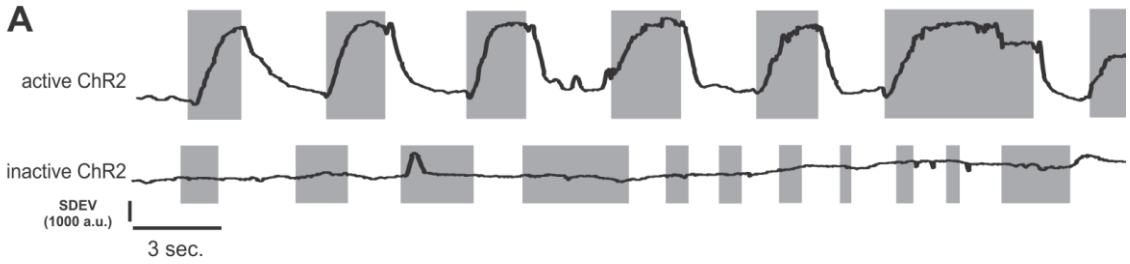

Figure 14. Stimulation of the gubernacular-oblique muscle group induces repetitive spicule thrusts and Ca^{2+} transients in the spicule protractor-anal depressor muscles. ***A***. Displacement of the spicule during and between brief blue light pulses. The grey regions indicate the time periods of blue light pulses. An image sequence of the male tail was captured for ~30 seconds when light pulses were applied repetitively. A region of interest (ROI) was placed at the base of a spicule, and the standard deviation of the pixel intensity within the ROI was obtained to indicate spicule displacement (see Experimental procedures). A representative trace for males that expressed active ChR2 (in the presence of all-*trans* retinal) is shown in the upper panel; a representative trace for males that expressed inactive ChR2 (in the absence of all-*trans* retinal) is shown in the lower panel. a.u. arbitrary units.

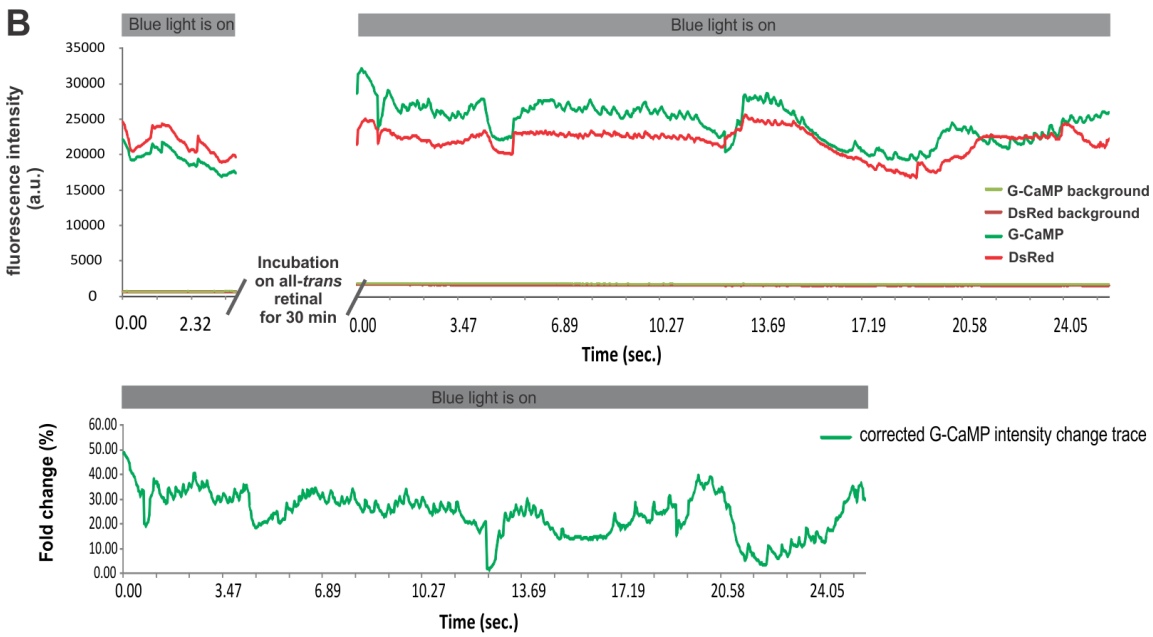

Figure 14 continued.
B. Raw fluorescence and corrected intensity traces for a representative male. Upper panel: raw fluorescence intensity traces in the protractor without gubernacular-oblique muscle stimulation for the baseline recording (with inactive ChR2), and during stimulation of active ChR2 on the gubernacular-oblique muscle group. Fluorescence intensity for both G-CaMP and DsRed channels are plotted, as well as the background fluorescence intensity for each channel. The periods that the light stimuli were applied are indicated by grey bars; the numbers on the X-axis indicate the time points since the onset of the most recent light stimulus; the 30 minute incubation is indicated on the X-axis. a.u. arbitrary units. Lower panel: corrected G-CaMP fold change trace during light stimulation.

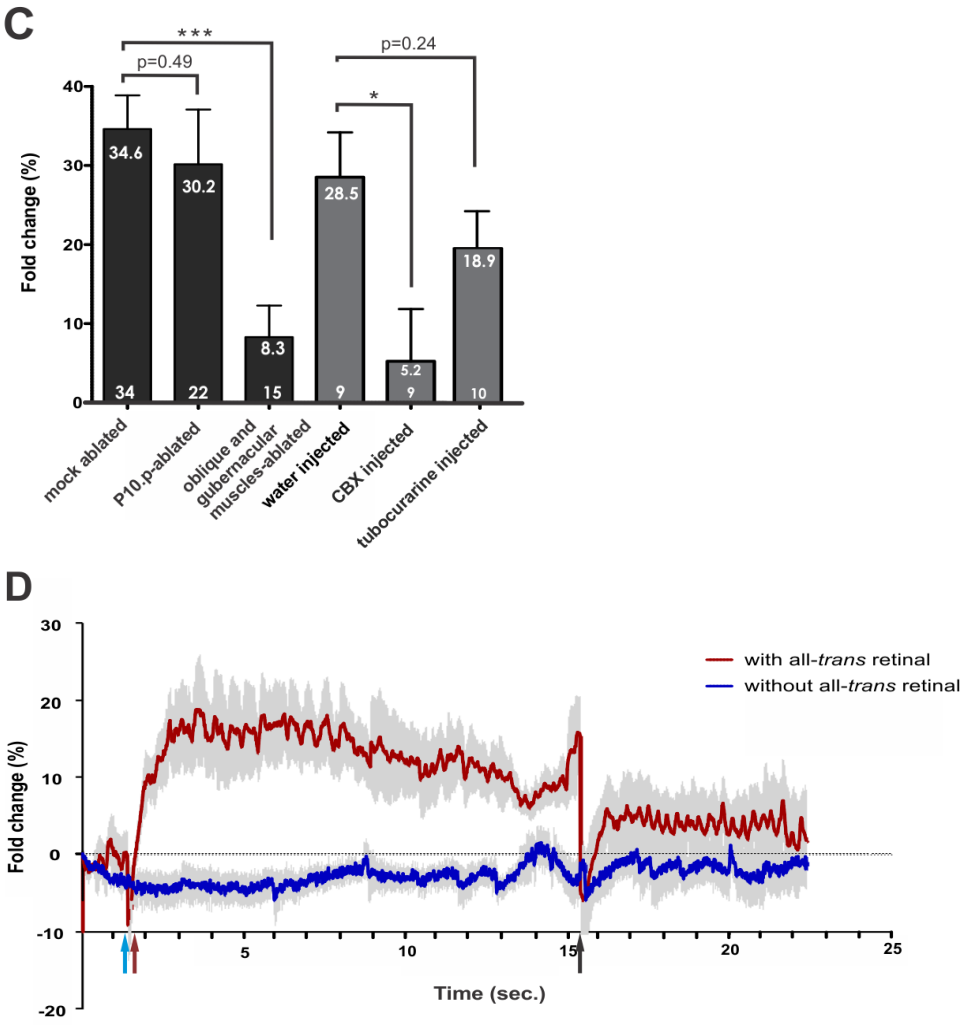

Figure 14 continued.
C. Maximal G-CaMP intensity changes in the protractors during light stimulation. The number of males assayed for each operation is listed at the bottom of each bar. The numbers below the top of the bars indicate the mean of maximal G-CaMP intensity changes of the population. Error bars indicate the standard error of the mean. Asterisk (***) indicate the *p* value <0.0005, (*) indicates the *p* value <0.05, calculated using the Mann-Whitney non-parametric test.
D. Average G-CaMP intensity changes before, during and after a pulse of blue light stimulation. The blue arrow indicates the beginning of light stimulation, the red arrow indicates the onset of G-CaMP intensity increase, and the black arrow indicates the end of light stimulation. The trace in red represents males with active ChR2. Males without active ChR2 are represented by the blue trace. The grey region around each curve represents the standard error of the mean. 4 males were measured for each trace.

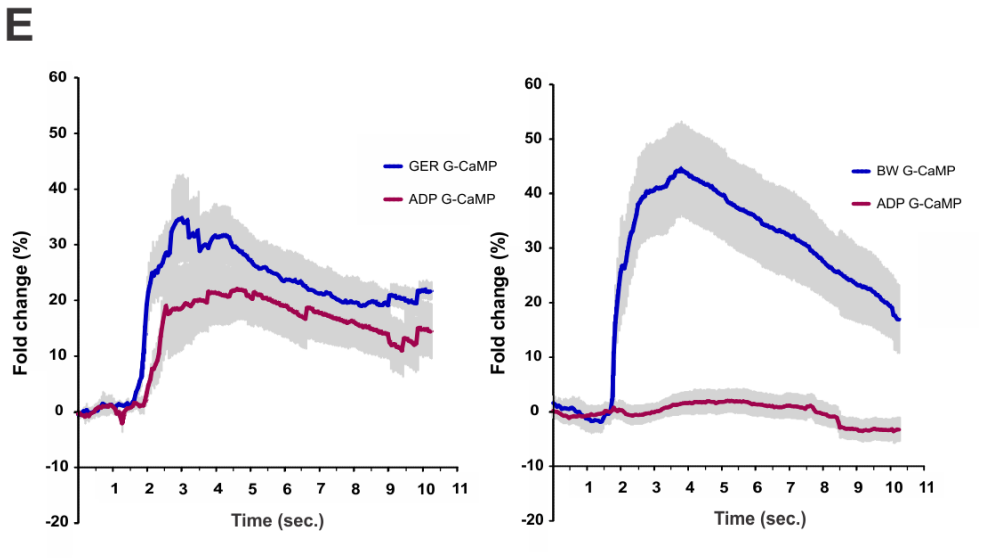

Figure 14 continued.
E. Averaged G-CaMP intensity traces in the anal depressor muscle (ADP), the gubernacular erector (GER) and a posterior body wall muscle (BW), upon laser microbeam stimulation of the gubernacular erector muscle or a body wall muscle in the male tail. Traces represent averaged G-CaMP fluorescence in specific muscles. The grey region around each trace represents the standard error of the mean. Left panel, the gubernacular erector on one side of the males was stimulated by a laser microbeam. The energy of the laser was adjusted to the lowest level that could elicit Ca^{2+} transients in the muscles. N=10 males. Right panel, a body wall muscle that has no gap junction to the protractor-anal depressor muscle group was stimulated by a laser microbeam. Ca^{2+} transients in the gubernacular erector and the anal depressor muscles were detected by using G-CaMP. Stimulation of the posterior body wall muscle failed to induce Ca^{2+} transients in the anal depressor muscle, indicating that in the left panel, Ca^{2+} transients in the anal depressor muscle were not caused by non-specific laser damage in the male tail. N=11 males.

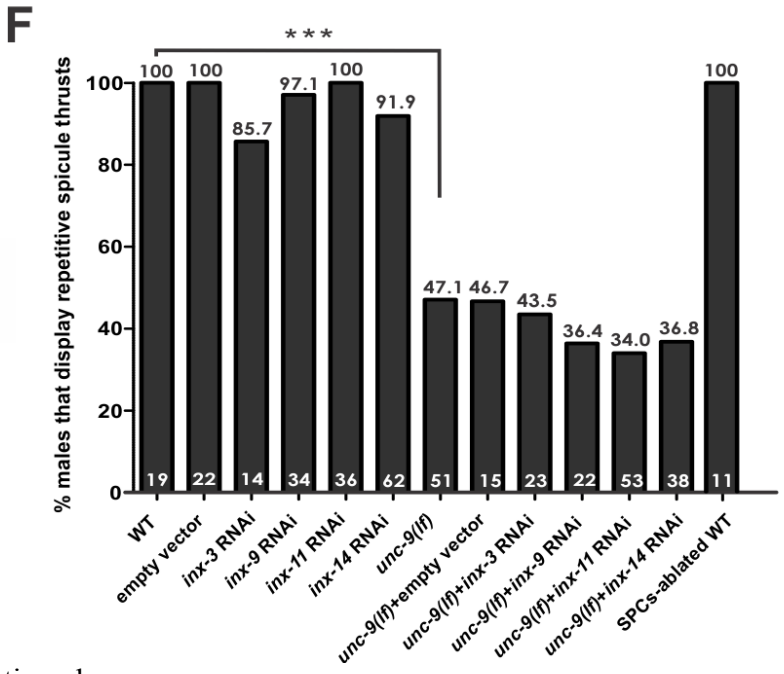

Figure 14 continued.
F. Effects of innexin mutant, RNAi of innexins and SPC ablation on ChR2-induced spicule thrusts. The *unc-9(lf)* males were compared to the wild type. The innexin RNAi strains with a wild-type genetic background were compared to the one that was fed with empty vector. The innexin RNAi strains with an *unc-9(lf)* genetic background were compared to the *unc-9(lf)* that was fed with empty vector-containing bacteria. The SPC-ablated wild type was compared to un-operated wild type. The numbers above the bars show the percentage of males that displayed rapid spicule thrusts upon light stimulation. The number of males assayed for each strain is listed within the bar. Asterisks (***) indicate the *p* value <0.0001, using the Fisher's exact test.

To detect Ca^{2+} level changes in the protractor-anal depressor muscles of intact males, I expressed a fluorescent Ca^{2+} indicator, called G-CaMP, using one of the *unc-103* promoter region (P*unc-103E*) (Nakai et al., 2001; Reiner et al., 2006; Gruninger et al., 2008). A red fluorescent protein DsRed, whose intensity does not change with Ca^{2+} level, was also expressed in the same set of cells under the same promoter region as an internal control (P*acr-18:ChR2::YFP* + P*unc-103E:G-CaMP* + P*unc-103E:DsRed*)

(Matz et al., 1999; Baird et al., 2000; Gruninger et al., 2008). The excitation spectrum of G-CaMP overlaps with ChR2 (Nakai et al., 2001; Zhang et al., 2008a). To determine the baseline of the G-CaMP/DsRed intensity ratio in the protractor-anal depressor muscles (in the absence of ChR2-mediated gubernacular-oblique muscle activation), males were first imaged under the blue excitation light prior to exposure to all-*trans* retinal. Afterwards, they were incubated on plates that contained all-*trans* retinal for 30 minutes and then reimaged again under the blue light. 30 minutes of incubation was sufficient to activate ChR2 for light stimulation (Fig. A-6). In the presence of all-*trans* retinal, I observed Ca^{2+} transients in correlation with light-induced contractions that caused muscle length changes (Fig. 14*B*, *C*; see Experimental procedures). In contrast, ChR2-expressing males that grew in the absence of all-*trans* retinal, did not display spicule thrusts or had Ca^{2+} transients in the protractor muscles (data not shown, n=30)

The P*acr-18* promoter region also drove transcription of ChR2::YFP in the HOA hook sensillum neuron (Fig. A-1). As described in a previous study, the hook neurons could also induce transient rhythmic spicule prodding behavior at the vulva independent of the p.c.s. neurons (Garcia et al., 2001). I then asked if the light-induced spicule protractor contractions and Ca^{2+} currents were results of activation of the HOA. I laser-ablated the hook sensillum precursor cells P10.p in the late L2 larval stage, so the adult males lack the whole sensillum structure. Similarly to the intact animals, the blue light still induce protractor contractions and Ca^{2+} currents in these males with a magnitude that was slightly, but not significantly, lower than the intact animals (Fig. 14*C*). This

suggests that under this experimental condition, HOA is not the major contributor for the Ca^{2+} currents recorded in the protractor-anal depressor muscles.

To determine whether the rhythmic contractions of the protractor-anal depressor muscles and the Ca^{2+}currents in these muscles were indeed a result of gubernacular-oblique muscle activity, I laser-ablated these muscles (the gubernacular erectors and retractors, the anterior and posterior obliques) in the L4 larval stage and asked if the males still display light-induced behaviors when they became adults. I found that the repetitive contractions of the protractor-anal depressor muscles were impaired, and the Ca^{2+} currents were greatly reduced in these males (Fig. 14*C*, $p<0.001$). I reasoned that although not eliminating the cells, ablating cell nuclei at the L4 larval stage severely impaired the physiology of these muscle cells and reduced the amount of ChR2 proteins on the remaining cell membranes. Thus, the cell corpses that remained after the operation could not be activated sufficiently to pass ions or signal molecules to the protractor-anal depressor muscles, and therefore no Ca^{2+}currents could be detected. In addition, the remaining activity I observed in the protractor-anal depressor muscles could also be due to the activation of the male-specific sphincter muscle and the spicule retractor muscles. In addition to the gubernacular-oblique muscle group, the upstream region of *acr-18* also drives transcription in these muscles (Fig. A-1). These muscles are also connected to the protractor-anal depressor muscles through electrical junctions (Male Wiring Project). Therefore, ChR2 expressed on these muscles might depolarize them and then contribute to the remaining activity of the protractor-anal depressor muscles. However, in an intact male, the sphincter and spicule retractor muscles are not

likely to be the major facilitators of the rhythmic spicule prodding behavior. Instead, their activities are more likely to be a result of activation of other male sex muscles, as they lack innervations from male-specific neurons (Male Wiring Project). I therefore conclude that activities of the gubernacular-oblique muscles can be relayed to the spicule protractor-anal depressor muscles probably through gap junction structures to induce rhythmic protractor contractions. In this way, when the p.c.s. neurons activate the gubernacular and oblique muscles to maintain male tail contact with the hermaphrodite's vulva, they also initiate the spicule prodding behavior.

Difference in the G-CaMP/DsRed intensity ratio under light stimulation when males were and were not incubated on all-*trans* retinal demonstrated that direct stimulating the gubernacular-oblique muscles could induce the anal depressor-protractor muscles activities. However, the temporal correlation between gubernacular-oblique muscle group depolarization and protractor-anal depressor muscle activities still needed to be determined. One way to assess the temporal correlation is to monitor Ca^{2+} levels continuously in the protractor muscles before, during and after a brief pulse of blue light stimulation of the gubernacular-oblique muscles. Using a Mosaic Imaging System (Andor™ Technology), I excited multiple regions simultaneously or sequentially without optically stimulating the whole field of view. I found that Ca^{2+} levels elevated gradually in the protractor muscles upon light-activation of the gubernacular-oblique muscles, and declined rapidly after the stimulation (Fig. 14*D*; see Experimental procedures). Alternatively, I stimulated the gubernacular erector muscles using a laser-induced muscle contraction method (Reiner et al., 1995). In males that had no ChR2

expressed, abrupt release of internal Ca^{2+} stores in the gubernacular erector muscle, via irradiating the muscle with a low-energy laser microbeam (440 nm), also elicited Ca^{2+} transients in the protractor-anal depressor muscles, causing rapid muscle contraction (Fig. 14*E*; see Experimental procedures). These results therefore suggest that activities of the gubernacular-oblique muscle group, initiated by the p.c.s. neurons, can be transmitted to the spicule protractor muscles directly, probably through gap junctions.

Gap junctions are required for information transmission from the gubernacular-oblique muscle group to the protractor muscles

To determine if gap junctions mediate the activity transmission between the gubernacular-oblique muscles and the protractor-anal depressor muscles, I asked whether the "muscle-induced muscle contraction" phenomenon could be suppressed by application of a gap junction inhibitor. I used Carbenoxolone (CBX), which has been shown to inhibit innexin function in invertebrates (Davidson and Baumgarten, 1988; Schneider and Stengl, 2006; Bao et al., 2007).I found that application of this drug reduced the light-induced Ca^{2+} transients in the protractor-anal depressor muscles (Fig. 14*C*), as well as the repetitive muscle contractions (data not shown). To the contrary, application of tubocurarine, which blocks both nicotine-sensitive and LEV-sensitive AChRs (Raizen et al., 1995; Ballivet et al., 1996; Fleming et al., 1997; Richmond and Jorgensen, 1999; Sattelle et al., 2002) and cause the males to be paralyzed, did not have an effect on either repetitive protractor contractions or Ca^{2+} transients in response to

light stimulation (Fig. 14*C*). These together indicate that functional gap junctions, instead of cholinergic synaptic transmission, are required for the gubernacular-oblique muscles activities to be relayed to the spicule protractor muscles.

To identify the innexin(s) that is important to the coupling of male sex muscles, I first determined innexin genes that are expressed in these muscles. The *C. elegans* genome encodes 25 innexin proteins. A high-resolution expression map of all innexins has been reported in the *C. elegans* hermaphrodite, and five innexins are expressed in the hermaphrodite sex muscles: *inx-3, inx-8, inx-9 inx-14* and *unc-9* (Altun et al., 2009). In addition, inx-11 was reported to be expressed in these muscles as well in another study (Starich et al., 2001). The hermaphrodite sex muscles are derived from the M-lineage, same as the male sex muscles (Sulston and Horvitz, 1977); hence, it is possible that these genes are also expressed in the male sex muscles. I inspected these genes' expression patterns in males by checking GFP expression under the control of their promoter regions (Altun et al., 2009). I found that *unc-9* and *inx-14* are expressed in multiple male-specific sex muscles and the sexually dimorphic anal depressor muscle. In addition, *unc-9* is also expressed in the SPC and the PCB neurons in the male tail (Fig. A-7).

To test if these innexins are used to transmit signals among the male sex muscles, I asked if the light-induced rapid thrusts of the spicules are affected when these genes are mutated or knocked-down by RNA interference (RNAi). Males that carry the *unc-9(e101)* loss-of-function allele have uncoordinated locomotion (Brenner, 1974), probably due to inhibited electrical coupling among body wall muscles, as well as

neuronal UNC-9 deficiency (Liu et al., 2006). I found that when ChR2 was expressed in the gubernacular-oblique muscles in these males, in the presence of all-*trans* retinal, only 47.1% of the males responded to the blue light with repetitive spicule thrusts (n=51, $p<0.0001$), compared to 100% of the wild type (n=19; Fig. 14*F*). To the contrary, light-induced spicule thrusts were not affected by RNAi of *inx-3*, *inx-9*, *inx-11* or *inx-14*. In addition, RNAi of these genes in the *unc-9(e101)* genetic background did not result in further inhibition of the behavior (Fig. 14*F*). In theory, the reduced spicule thrusts can be resulted from a deficiency of UNC-9 in the SPC and PCB neurons. I addressed this possibility by first asking if these neurons are required for blue light-induced spicule thrusts. I reasoned that the PCB neurons do not make synapse or gap junction to the protractors; as a result, for the PCBs to mediate the "muscle-induced muscle contraction" phenomenon, they have to send signals through the SPC neurons, which innervate the protractors. Therefore, I can test whether the PCB and SPC neurons are required for the "muscle-induced muscle contraction" phenomenon by asking if light-stimulated gubernacular-oblique muscle activity can still induce spicule thrusts when the SPC neurons are ablated. I found that ablation of the SPC neurons did not have an effect (Fig. 14*F*), suggesting that neither pair of these neurons is required and therefore a deficiency of UNC-9 in these neurons is not likely to cause reduced light-induced spicule thrusts. Taken together, these data suggest that the UNC-9 innexin is used in some of the gap junctions that mediate the direct signal transmission from the gubernacular-oblique muscles to the protractor-anal depressor muscles. Identification of additional gap junction subunits awaits further study.

The SPC neurons are necessary but not sufficient to trigger sustained protractor contraction

At the vulva, the rhythmic shallow spicule thrusts cease when prolonged spicule protraction occurs. It remains unknown what mechanism underlines the switch between these two states of the protractor muscle contractions (rhythmic and prolonged). A previous study has shown that the cholinergic SPC neurons are required for rhythmic contractions to switch to the prolonged contraction (Garcia et al., 2001). I hypothesized that during rhythmic contractions, when ionotropic AChRs on the protractors are activated by ACh secreted by the SPC neurons, stronger depolarization then induces prolonged spicule protraction. If this is the case, depolarizing the protractor muscles directly by light-activated ChR2 should, in theory, cause prolonged spicule protraction.

To test this theory, I expressed ChR2 in all muscles, using the P*lev-11* promoter (P*lev-11:ChR2::YFP*) (Gruninger et al., 2006). When grown on all-*trans* retinal, all transgenic males displayed strong body wall muscle contraction in response to the blue light as a result of activation of the body wall muscle ChR2. However, only 5% of the males showed tonic contraction of the protractor muscles (n=20; Fig. 15). The rest of the population responded to the blue light with shallow repetitive protractor contractions with the tips of their spicules slightly protruding from the cloacal opening. Similarly, when I expressed ChR2 only in all the male-specific sex muscles, using the P*unc-103E* promoter (P*unc-103E:ChR2::YFP*), only 18% of these males fully protracted their

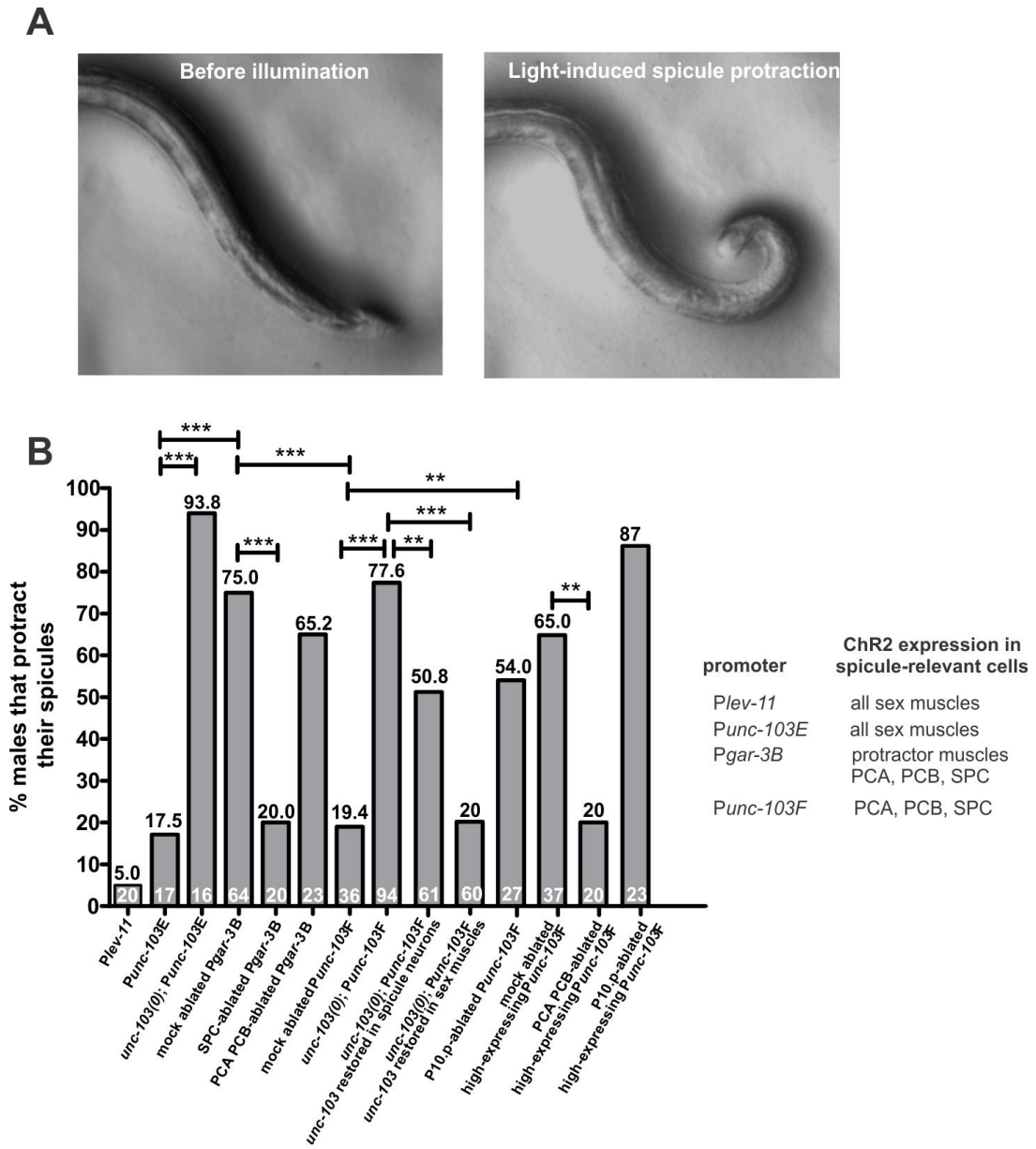

Figure 15. Simulation of ChR2 in different spicule circuit components.
A. Before illumination, the male that expresses P*gar-3:ChR2::YFP* holds his spicules (arrow head) inside of his tail. The blue light induces sustained spicule (arrow head) protraction of this male.
B. Sustained spicule protraction induced by simulation of ChR2 in different circuit components. The numbers on the vertical axis represent the percentage of males that protraction their spicules when different circuit components are activated by light-stimulated ChR2. The numbers above the bars are the actual percentage of males that protracted in response to light. The numbers of males assayed for each strain are listed within the bars. Asterisks (***) indicate the *p* value<0.0001, (**) indicate the *p* value<0.001, calculated using the Fisher's exact test.

spicules (n=17; Fig. 15). Light could only induce repetitive shallow protractor contractions in the rest of these males. Given that ChR2 was functioning, and was sufficient to depolarize the body wall muscles, I reasoned there might be some negative regulatory molecules on the protractor keeping these muscles from contracting tonically, and it is likely that the SPC neurons activity is still required for rhythmic contractions to transform into tonic contraction.

To address if negative regulator molecules suppress the protractors from fully contracting, I introduced a null allele of the *unc-103* gene into the P*unc-103E:ChR2::YFP*-expressing males. The *unc-103* gene encodes an ERG-like K^+ channels. The human ERG K^+ channels have been studied mostly in cardiac muscles for its role in regulating rhythmicity (Vandenberg et al., 2001; Sanguinetti and Tristani-Firouzi, 2006). Mutations in this gene result in inherited long QT syndrome (Curran et al., 1995; Pond and Nerbonne, 2001). In *C. elegans*, UNC-103 K^+ channels are considered to negatively regulate the cell excitability, and *unc-103* deletion males spontaneously protract their spicules in the absence of mating stimulation (Garcia and Sternberg, 2003; Reiner et al., 2006). About 94% of the *unc-103(0)* males that had ChR2 expressed in their sex muscles responded to the blue light with tonic protracted spicules (n=16; Fig. 15). This suggests that the functional UNC-103 K^+ channel on the protractor muscles is used prevent depolarized spicule protractors from tonically contracting, when the SPC neurons are not activated.

To address whether in wild-type males, activation of the SPC neurons can transform the protractor rhythmic contractions into tonic contraction, I expressed ChR2

in the SPC neurons in addition to the sex muscles by using the promoter region of the *unc-63* gene (P*unc-63*) described in Chapter IV. About 57% of the males that expressed the P*unc-63:ChR2::YFP* construct fully protracted their spicules under the blue light, significantly higher than 18% of the P*unc-103E:ChR2:YFP* males (n=30, p=0.0139). In addition, I used the P*gar-3*B promoter region described in Chapter III, to express ChR2 in the SPC neurons and two of the p.c.s. neurons (PCA and PCB), in addition to the spicule protractors and the anal depressor muscle (P*gar-3*B*:ChR2::YFP*). I found 75% of the males that expressed this construct protracted their spicules under the blue light (n=64, p<0.0001; Fig. 15). Ablation of the PCA and PCB neurons did not significantly affect the light-induced spicule protraction (Fig. 15; n=23), suggesting that although solely depolarizing the protractor muscles does not cause these muscles to tonically contract, activation of the SPC neurons together with the muscle activity induces the sustained contraction. Consistent with this, when I laser-ablated the SPC neurons in these transgenic males, I found the percentage of males could tonically protract spicules under the blue light reduced to 20% (Fig. 15; n=20), indicating an essential role of the SPC neurons in making the switch.

Signals from the p.c.s. and hook sensillum are integrated to promote sustained spicule protraction

If activation of the p.c.s. neurons induces rhythmic spicule protractor contraction, and activation of the SPC neurons switches the contractile state to tonic contraction, then

artificially activating these neurons using light-activated ChR2 should cause sustained spicule protraction. Using a P*acr-12* promoter that was described in Chapter IV, I expressed ChR2 in the SPC and one of the p.c.s. neurons (P*acr-12:ChR2::YFP*). Only 39% of the transgenic males protracted their spicules under the blue light (n=23). I reasoned that since only one of the three p.c.s. neurons were stimulated, this might not be as efficient as activating all three neurons.

I then used one of the *unc-103* gene promoter regions (P*unc-103F*) to express ChR2 in the PCA, PCB, and SPC neurons (Reiner et al., 2006). Since males that had either two of the p.c.s. neurons could still prod their spicules at the vulva and eventually insert their spicules, I expected more males that expressed the P*unc-103F:ChR2::YFP* construct to protract their spicules under light. To my surprise, only 19% of the P*unc-103F:ChR2::YFP* expressing males showed sustained spicule protraction in response to the blue light (Fig. 15; n=36). When I preselected males that displayed a very high expression of P*unc-103F:ChR2::YFP*, 65% of these biased males displayed spicule protraction after light stimulation (Fig. 15; n=37). Ablation of the PCA and PCB neurons reduced the percentage of males that responded to the light to 20% (Fig. 15; n=20, p=0.002), indicating that in the absence of PCA and PCB, activation of SPC alone is not sufficient to induce tonic muscle contraction.

The low efficiency of P*unc-103F:ChR2::YFP*-induced spicule protraction in unbiased males suggested that, the spicule-prodding/protraction neurons have intrinsic properties, which to overcome, require high levels of ChR2 stimulation. When the P*unc-103F:ChR2::YFP* construct was introduced into the *unc-103(0)* males, 77.6% of the

population fully protracted their spicules in response to the blue light (n=94, Fig. 15). The *unc-103* gene is expressed in both spicule-associated neurons and the male sex muscles (Gruniner et al., 2006; Reiner et al., 2006). I found that restoring functional UNC-103 in the sex muscles could reestablish these males' resistance to light-stimulation; however, expressing *unc-103* in the spicule-associated neurons can also restore some resistance to light stimulation (Fig. 15; n=26, $p<0.0001$). Thus in wild type, the UNC-103 channel likely regulates the excitability threshold of neurons, in addition to muscles.

However, this still does not address the question, 'why do p.c.s. and SPC neurons promote spicule insertion at the vulva, but not efficiently under artificial non-mating conditions?' The male copulation is a complex behavior. Multiple sensory inputs probably need to be integrated for the behavior to be executed coordinately, whereas for the P*unc-103F:ChR2::YFP*-induced behavior, only the SPC, PCA, and PCB neurons are activated. I hypothesized that activities of these neurons were attenuated by other neurons, and this attenuation would be removed when the vulva is sensed. It was reported in previous research that the hook sensillum could sense the vulval signal and induce the spicule prodding behavior. When the hook sensillum precursor cell, P10.p is laser-ablated, the operated males no longer sense the general area of the vulva, but instead randomly prod the cuticle of the hermaphrodite with their spicules (Liu and Sternberg, 1995). This suggests that in intact males, the cells derived from P10.p attenuate spicule protractor activity until the male circuit senses the vulva. The P10.p precursor cell gives rise to the hook structural cells, HOA and HOB sensory neurons,

and the PVZ motor neuron. The PVZ does not directly synapse the spicule-relevant neurons, but the HOA and HOB neurons make direct chemical and electrical connections to the p.c.s. and SPC neurons (Male Wiring Project). Thus, it is possible that HOA and HOB negatively regulate spicule activity until the vulva is sensed.

Laser-ablation of the hook sensillum precursor cell P10.p resulted in 54% of the un-biased males (Fig. 15; n=26), and 87% of the high-expressing males (Fig. 15; n=23), to protracted their spicules under the blue light. The incidence of ChR2-induced protraction was higher for the operated males than the non-operated males, in both cases. Though for biased P*unc-103F:ChR2::YFP* high-expressing males, the increase was not statistically different over the already high protracting non-ablated males (p=0.08), the difference was statistically significant (p=0.004) for the un-biased males. Interestingly, under the blue light, ChR2 was not expressed in the hook sensillum to activate it. This indicates that the hook neurons are active to attenuate the activities of the SPC, PCA, and PCB neurons in the absence of the vulva signal.

Chapter summary

In this Chapter, I identified the cellular mechanisms that underlie the bi-stable state of the spicule protractor muscle contraction (rhythmic shallow contractions and prolonged contraction). It has been suggested in a previous study that the rhythmic contractions are induced by the p.c.s. neurons and the sustained contraction requires the SPC cholinergic neurons (Garcia et al., 2001). Since the protractors are only innervated

by the SPC, but not the p.c.s. neurons, it remained unknown what neuronal mechanism the circuit uses to give rise to two distinct types of muscle contractions.

Guided by the physical connections of the male circuit revealed by the Male Wiring Project, I explored functional connections that are important for generating these motor outputs. In a carefully designed experimental set, I found that selectively depolarizing the gubernacular-oblique muscle group, which is innervated by the p.c.s. neurons, could instantaneously induce repetitive shallow spicule thrusts and Ca^{2+} currents in the protractor muscles. Given the gubernacular-oblique muscle group is connected to the protractors via gap junctions, this suggests that for the p.c.s. neurons to induce rhythmic protractor contraction, they activate the gubernacular-oblique muscles, and the activity then is relayed to the protractors via gap junctions.

I also demonstrated that for the male circuit to switch from rhythmic spicule protraction to prolonged spicule protraction, multiple sensory inputs need to be integrated by the nervous system. By expressing the light-gated cation channel ChR2 in specific circuit components, in conjunction with laser-ablation of a particular set of cells, I could ask which sensory neurons are necessary and sufficient to promote prolonged spicule protraction. I found that for the circuit to give rise to sustained protractor contraction most efficiently, the p.c.s. neurons and the SPC neurons need to be activated, and the hook sensillum neurons need to be inactivated. This suggests a scenario that before the males locate the vulva, the hook neurons are active to suppress spicule activity until they recognize the vulva and remove the inhibition. The vulva signal also activates the p.c.s. neurons to induce the spicule prodding behavior, until partial

penetration of vulva triggers the SPC neurons, whose activity in conjunction with the continuing p.c.s. activities then promote the sustained spicule insertion.

CHAPTER VI

SUMMARY OF EXPERIMENTS AND DISCUSSION*

Summary of experimental results

The *C. elegans* males utilize a couple of neurons and muscles to accomplish the reproductive behavior with stereotyped but complex motor patterns. The purpose of this study was to elucidate the cellular and molecular mechanisms the male circuit uses to give rise to different motor outputs and coordinate them into a coherent behavioral sequence. My study demonstrated that by taking advantage of different types of circuit connections and diverse receptor types, the cholinergic copulation circuit regulates both the rhythmic spicule thrusts and the sustained spicule insertion, and coordinate these two motor outputs to facilitate the mating success.

I found that once the postcloacal sensilla (p.c.s.) neurons recognize the vulva, they continuously function to maintain the precise male tail position over the vulval slit. Two of the three p.c.s. neurons are cholinergic, and they are likely to facilitate the male vulval contact through innervating the posterior male tail muscles by activating the L-AChR and the ACR-16 nAChR on these muscles. Additionally, I found that the activity

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* Part of this chapter is reprinted with permission from "G{alpha}q-Coupled Muscarinic Acetylcholine Receptors Enhance Nicotinic Acetylcholine Receptor Signaling in Caenorhabditis elegans Mating Behavior" by Liu Y, LeBoeuf B, Garcia LR, 2007, *Journal of Neuroscience*, 27, 1411 – 1421. Copyright © 2007 by Society for Neuroscience.

of the oblique muscles and the gubernacular muscles, which are connected via gap junctions, can be conducted to the spicule protractor muscles instantaneously to induce Ca^{2+} currents and shallow rhythmic contractions of these muscles. Gap junction structures are also found to connect the gubernacular-oblique muscles with the spicule protractors. This suggests that the p.c.s. neurons probably trigger rhythmic spicule thrusts by utilizing the electrical junctions between these muscles.

The switch from rhythmic spicule prodding to tonic spicule insertion is under strict regulation. When I asked which neurons activities were necessary and sufficient to cause sustained spicule protraction, I found that although the SPC is the ultimate trigger of the behavioral change, this behavior also requires the activities of the p.c.s. neurons. In addition, it requires the hook sensillum, which senses the vulva, to be inactive. By integrating all these sensory cues, the circuit can control the timing of the spicule insertion to insure the success.

Understanding the basic operational logic of the copulation circuit is likely to help us to address questions such as how modulatory signaling pathways influence the circuit activity. In the first part of this study, I demonstrated that a $G\alpha_q$-coupled mAChR-mediated signaling pathway is used to facilitate the male mating efficiency, probably via enhancing the motor output of the ionotropic AChRs-mediated synaptic transmission. The ionotropic AChRs were only known to promote the sustained spicule protraction (Garcia et al., 2001). Therefore it was not obvious why the mutant males, which had abolished mAChR signaling, could eventually penetrate the vulva during mating, but they could not accomplish it as efficiently as the wild type. Having an understanding of

how ionotropic AChRs are utilized to facilitate the male vulval contact put this finding into a better context. It is possible that by enhancing the ionotropic AChRs signaling in the oblique muscles as well as in the protractors, the $G\alpha_q$/ mAChR signaling pathway facilitates the male vulval contact so that the males can insert their spicules more efficiently.

The cholinergic neuromuscular junctions between the postcloacal sensilla neurons and the oblique muscles are used to maintain the male tail position at the vulva

The *C. elegans* males copulate with the opposite sex, hermaphrodites, by inserting their copulatory spicules into the hermaphrodites' vulvas to facilitate sperm transfer (Liu and Sternberg, 1995). The males cannot penetrate the hermaphrodite's vulva immediately, unless the hermaphrodite has experienced extended egg-laying and her vulva is dilated (the hermaphrodites with dilated vulvas are usually 2 days into their adulthood or older). Instead, the males prod their spicules at the vulva rhythmically, as an attempt to breach the slit, and then partial spicule insertion is sensed by the SPC spicule neurons to trigger full spicule protraction (Garcia et al., 2001). When a male attempts to mate, the hermaphrodite tries to move away, since for the sperm-carrying hermaphrodite, mating is not required for reproduction. Thus, an essential step for the males to successfully mate is to sustain precise contact between their cloaca opening and the hermaphrodite's vulva, so that their insertion attempt is prolonged for them to penetrate the vulva.

The first step to achieve vulval contact is to recognize the vulva, or to localize it. This requires the combined functions of two sensory organs of the male, a bilateral set of postcloacal sensilla, which contains three pairs of sensory-motor neurons (PCA, PCB and PCC), and the hook sensillum that contains the HOA and HOB sensory neurons. It was suggested that the p.c.s. neurons might be mechanosensory, as their sensory endings are encased in cuticle; whereas the HOA and HOB might be both mechanosensory and chemosensory, since their sensory endings are opened to the environment (Sulston et al., 1980). Males that lost function of all p.c.s. neurons after laser-ablation of these neurons could stop at the general area of the vulva, but could not position their tail precisely over the vulva slit and insert their spicules(Liu and Sternberg, 1995; Garcia et al., 2001). In contrast, males that did not have the functional hook sensillum displayed reduced efficiency in recognize the vulva; however, these males could still locate the vulva and insert spicules at a lower efficiency (Liu and Sternberg, 1995; Barr and Sternberg, 1999; Yu et al., 2003; Jauregui and Barr, 2005; Peden and Barr, 2005; Bae et al., 2008).

In this study, males that had any pair of the p.c.s. neurons laser-ablated could localize the vulva, suggesting that males can utilize any two pairs of these neurons to position their tail at the vulva. However, if males that lack any pair of the p.c.s. neurons could not insert their spicules immediately, they moved off the vulva sooner than the intact animals. This suggests that after the initial recognition, these neurons are continuously used to maintain the male's position over the hermaphrodite's vulva.

Mutant males that lack the functional L-AChR and ACR-16 nAChR showed similar vulval contact defect as the single pair p.c.s. neuron-ablated males. These

receptors are both expressed on a couple of male-specific sex muscles, including the spicule protractors, the gubernacular muscles and the oblique muscles. Among these muscles, the gubernacular and the oblique muscles are innervated by the cholinergic p.c.s. neurons (PCB and PCC), as well as the non-cholinergic p.c.s. neurons (PCAs) (Male Wiring Project) (Garcia et al., 2001). Therefore, it is possible that the maintenance of male tail position at the vulva is regulated by direct synaptic transmission between the p.c.s. neurons and these male-specific muscles (Fig. 16). This is supported by the fact that the oblique muscle-ablated males could not maintain their tail position at the vulva as well as intact animals. In addition, it is further supported by the fact that restoring functional L-AChR back to the male sex muscles reversed the vulval contact defect in the ionotropic AChRs double mutant males. The oblique muscles lie dorsal-ventrally at the male tail slightly posterior to the cloacal opening, and they are attached to the body wall. When they contract, the posterior tail region curls ventrally, probably as a result of their dorsal-ventral oriented single sarcomeres (Lints and Hall, 2009). I speculate that when males are moving backward along the hermaphrodite cuticle, changes in their tail curvature might redirect the force of the tail from lateral scanning to downward pressing (Fig. 16), therefore stabilizing the male at the vulva.

The gubernacular muscles, also innervated by the p.c.s. neurons, are not required for prolonged vulval contact. However, they seem to facilitate sperm transfer, as ablating these muscles resulted in males that could not efficiently release their sperms after spicule insertion. The gubernacular muscles are attached to the gubernaculum, a cuticle structure locates adjacent to the spicules. I reason that when these muscles contract, they

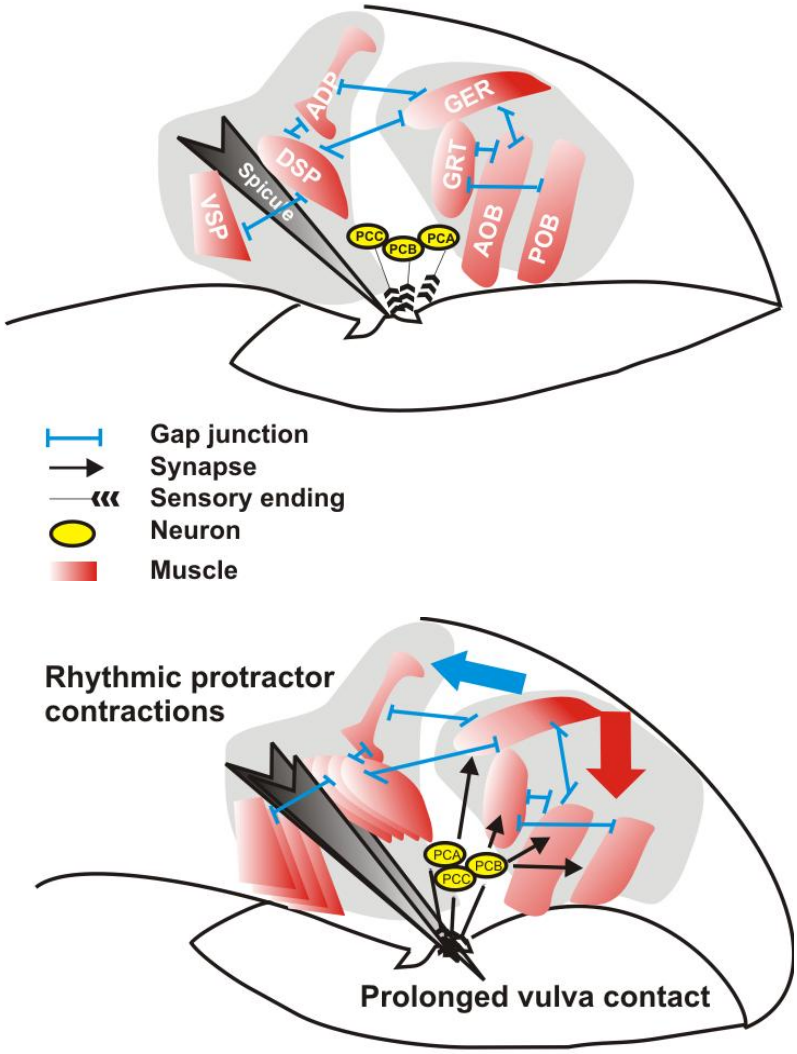

Figure 16. Model of the coupling of prolonged vulval contact with rhythmic protractor contractions.
Muscle abbreviations: DSP: dorsal spicule protractor; VSP: ventral spicule protractor; ADP: anal depressor; GER: gubernacular erector; GRT: gubernacular retractor; AOB: anterior oblique; POB: posterior oblique;
Cartoon depicting changes in male tail muscles upon vulval contact. Upper panel depicts the relative locations of the male sex muscles and the electrical junctions between them. The lower panel depicts the shortening of the AOB and POB muscles causing a curvature in the posterior male tail (red arrow) upon chemical stimulation from the PCA, PCB and PCC neurons. Stimulated GER muscles relay their signals to the spicule protractor muscles causing rhythmic contractions (blue arrow).

not only lift the gubernaculum, but also lift other adjacent tissues to allow sperm in the distal vas deferens to drain out.

The p.c.s. neurons coordinate both vulval contact and rhythmic spicule shallow thrusts simultaneously via gap junctions

For wild-type males, contact with the vulva and rhythmic spicule shallow thrusts always occur simultaneously. Since I rarely observed either behavior happens independently, I speculated they must share some common neuronal regulation mechanisms. The rhythmic spicule thrusts are results of repetitive protractor muscles contractions. My results showed that activity in the oblique and gubernacular muscles can be relayed instantaneously to the anal depressor muscle and the protractor muscles to induce Ca^{2+} currents and cause repetitive contraction of these muscles. The intercellular communication is likely mediated by the electrical couplings between these two clusters of muscles (Male Wiring Project; Fig.s 11, 14 and 16). Therefore, by utilizing gap junctions in the circuit, two different motor outputs are coordinated to occur simultaneously. Since vulva penetration requires both precise vulval contact and repetitive spicules thrusts at the vulva slit, the circuit maximizes the incidence of success by adopting these two motor patterns.

Although connected by gap junctions, different motor outputs are generated separately by the gubernacular-oblique muscle group and the anal depressor-protractor muscle group. I observed that, when males rhythmically prodded their spicules at the

vulva, they maintained a stable tail curvature. This suggests that the oblique muscles do not undergo high frequency contractions like the protractors. I speculate that this can be a result of either of two mechanisms: 1) ion current, such as Ca^{2+}, is directly conducted from the oblique-gubernacular muscle group to the protractors, however, it causes rhythmic contraction instead of tonic contraction of the protractors because of the specific property of the muscle motor proteins; 2) the signal molecules that pass from the oblique-gubernacular muscle group to the protractors via gap junctions, induce regenerative Ca^{2+} current in these muscles to cause rhythmic contractions. I favor the later, since it is consistent with the observation from a previous study that the ryanodine receptor Ca^{2+} channel gene *unc-68* is required for the rhythmic protractor contraction, but not the sustained contraction (Garcia et al., 2001).

In adult *C. elegans*, gap junctions are distributed extensively throughout the nervous system. Based on serial transmission electronic microscopy images and gap junction protein expression patterns, the gap junction structures are not limited between neurons or between muscles, but also connect neuron and muscle cells (White et al., 1986)(Male Wiring Project). These electrical connections probably play important roles in regulating animal behaviors. The *C. elegans* gap junctions have been reported to mediate Ca^{2+} current propagation throughout the intestine cells and the body wall muscles, and a few studies also indicate their roles in synchronizing neuron activities (Liu et al., 2006; Chen et al., 2007; Peters et al., 2007; Macosko et al., 2009). However, the functional importance of the majority of gap junctions in the *C. elegans* nervous system remains largely unknown.

The understanding of how the p.c.s. neurons promote prolonged vulval contact and rhythmic spicule thrusts will enable us to ask what cellular and molecular changes occur between closely related nematode species for them to have different behavioral patterns during mating. The cells in the *C. remanei* male tail form almost exactly the same patterns as the cells in the *C. elegans* male tail. However, unlike in *C. elegans,* the equivalent cells of the cholinergic p.c.s. neurons in *C. remanei* do not induce rhythmic protractor muscle contractions. Instead, they sense the vulva then induce sustained spicule protraction immediately (Garcia et al., 2007). Like in *C. elegans*, the *C. remanei* spicule circuit also utilizes ACh and ionotropic AChRs to promote sustained spicule protractor contraction (Garcia unpublished data). These observations raise the question whether the equivalent cells of the p.c.s. neurons in *C. remanei* could synapse directly to the spicule protractors to induce immediate spicule insertion in response to the vulva? In addition, one can ask if the patterns of spicule activity that is featured by the hermaphroditic species can be induced by just rewiring synapses from the p.c.s. neurons to the gubernacular-oblique muscle group. Answers to these questions will help us to understand whether and how differences in circuit connectivity contribute to diverged behavioral patterns between closely-related species. In *C. elegans*, it has been demonstrated that the patterns of neuronal connectivity can be regulated by a few genes (Hallam and Jin, 1998; Hallam et al., 2002). It is possible when a species evolves from its ancestor, changes in a few key genes that determine the circuit connections can dramatically change the behavioral patterns.

Sustained protractor muscle contraction requires integration of multiple sensory inputs

The core spicule circuit (the SPC neurons, the p.c.s. neurons and the spicule protractor muscles) is connected to other parts of the nervous system via extensive synapses and gap junctions (Male Wiring Project). Thus it is likely that activities in other behavioral circuits can cause non-specific activities (noise) in the spicule circuit when the male is not exposed to stimulation that induces the mating behavior.

I found that the circuit uses a "coincidence detector"-like mechanism to prevent the males from protracting their spicules into non-vulval orifices, or from responding to random environmental stimuli. In other words, the male protracts his spicules only if multiple signals indicate that he is in good contact with the vulva and ready to penetrate it. I uncovered this mechanism by using the light-gated cation channel channelrhodopsin (ChR2) to selectively activate different components of the male circuit. Activation of excitatory cells by ChR2 is not equivalent to the activation caused by synaptic transmission or electrical junctions in behaving animals, but these experiments enable us to see the capability of the spicule circuit to respond to various inputs with differential behavior outputs.

I found that at least three input signals have to be integrated by the protractor muscles for them to fully contract with the highest probability: removal of the negative input from the pre-cloacal sensillum neurons (HOA and HOB) in response to vulva contact; continuous activity of the p.c.s. neurons, which indicates the male tail is still

over the vulva; and activation of the positive input from the SPC cholinergic proprioceptive sensory-motor neurons, which directly synapse the protractors (Fig. 17). Among these, it is interesting that in the absence of vulva signal, the hook neurons suppress the spicule protractor activity. This suggests these neurons are constitutively active until the vulval signal suppresses their activity and releases the negative regulation from these neurons. At the same time, the p.c.s. neurons are also stimulated

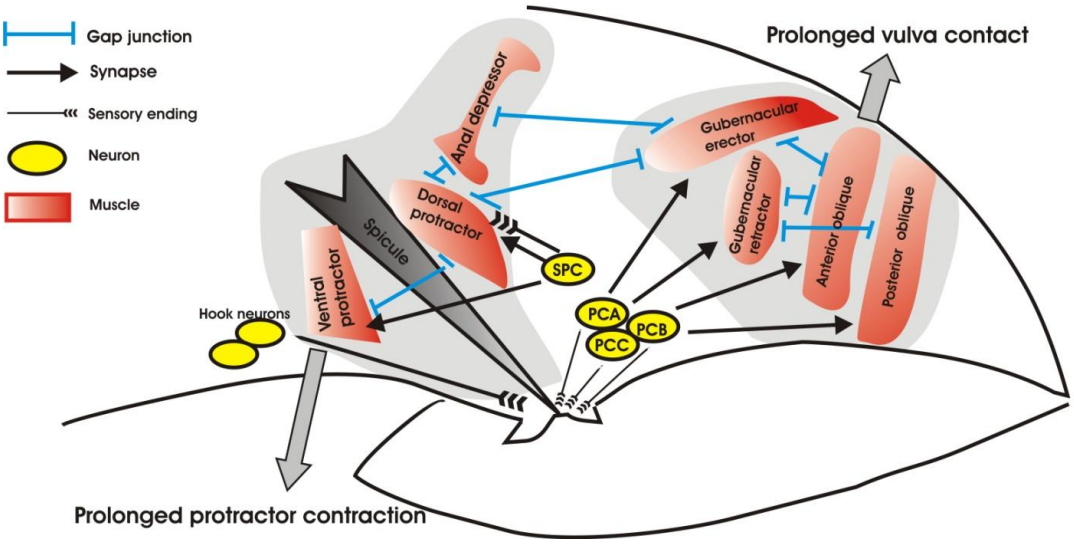

Figure 17. Integration of multiple sensory inputs is required to trigger tonic spicule full protraction.
The SPC neurons have sensory ending-like dendrites attached to the dorsal spicule protractor muscles. The hook neurons send their sensory processes to the hook structure anterior to the cloacal opening. The p.c.s. neurons send sensory ending to the post-cloacal area. Activation of the SPC neurons and the p.c.s. neurons facilitates tonic spicule protraction, whereas the hood neurons constitutively inhibit spicule protractor activity until they sense the vulva.

by the vulval signal. They secrete neurotransmitters, such as ACh, to induce rhythmic spicule thrusts. Eventually, partial spicule penetration triggers the SPC neuron activity, through their proprioceptive sensory endings, to release ACh onto the protractor muscles. ACh-activated nAChR on these muscles acts additively with the intracellular regenerative Ca^{2+} currents to switch the oscillating contractions to the tonic contraction.

The male copulation circuit has a high threshold to be stimulated, so only when all sensory inputs are detected "coincidently", will the circuit respond maximally to give rise to spicule protraction. I propose that the ERG-like K^+ channel UNC-103 is used by the circuit to set up such a high threshold. My results showed that when functional UNC-103 is absent from the circuit, the "coincidence detector" mechanism collapses, and the circuit then respond to stimulus that is not normally sufficient to induce spicule protraction in intact animals. This also helps to explain why the *unc-103(0)* mutant males display a fictive mating behavior spontaneously in the absence of hermaphrodite, and why during mating, they attempt to prematurely protract spicules at the hermaphrodite vulva before partial penetration is achieved (Garcia and Sternberg, 2003).

The *unc-103* gene is also expressed in a number of cells in *C. elegans*, including the body wall muscles and the ventral cord neurons that regulate the locomotion behavior, as well as the intestinal muscles that control defecation. Interestingly, in contrast to the spicule protraction defect, the *unc-103(0)* mutant worms, males or hermaphrodites, have no obvious or slight behavioral defects in most of behaviors, including locomotion and defecation (Garcia and Sternberg, 2003; Reiner et al., 2006). However, the *unc-103(0)* mutant hermaphrodites have obvious hyper active egg laying

behavior under non-optimal conditions (Reiner et al., 2006). This suggests that the UNC-103 function is required in circuits that regulate the reproductive behaviors to suppress the circuit excitability. I infer that for the reproductive behaviors, since the timing of the behavior needs to be more strictly regulated, the high threshold set up by the UNC-103 K^+ channel insures that only when multiple inputs are present the behavior occurs.

The model I just described showcases how the *C. elegans* nervous system uses simple cellular and molecular mechanisms to coordinate behavioral sequences. These mechanisms are likely to share common principles with what is underpinning behavioral coordination of more complex organisms. For example, in vertebrates, it has been suggested that the low excitability of the striatal neurons play an important role in prevent unintended locomotion. The central pattern generators in the spinal cord generate the locomotion (Grillner and Jessell, 2009). These networks remain silent, as they are tonically inhibited by the pallidum region of the basal ganglia. This inhibition can be removed when the pallidum receives inhibitory input from the striatum. The striatum has a low excitability due to the inward rectifier K^+ current (Nisenbaum and Wilson, 1995; Mermelstein et al., 1998; Kreitzer and Malenka, 2008). Therefore, the striatum only gets activated when it receive strong activation from the thalamus or the cortex, where relevant sensory inputs are integrated. Using this mechanism, the animal adapts its locomotion properly in response to environmental conditions (Grillner et al., 2008).

A $G\alpha_q$/mAChR signaling pathway facilitates the male mating efficiency via enhancing the ionotropic AChRs-mediated synaptic transmission

Activities of the male copulation circuit are not only under modulation of negative regulators, such as UNC-103, but also under control of positive regulators, such as the GAR-3 mAChR.

In virgin males, ACh secreted from the SPC neurons and the PCB neurons is required for the wild-type dose-response to LEV. As showed in Chapters III and IV, the LEV-sensitive nAChR (L-AChR) is only expressed on the male sex muscles that are postsynaptic to these neurons. This indicates that, though these muscles are capable of expressing functional L-AChR in the absence of presynaptic neurons, either the abundance or the signaling capacity of these L-AChR receptors must be modulated by their presynaptic partners.

I found that a signaling pathway mediated by the $G\alpha_q$-coupled mAChR, GAR-3, is required for the wild-type behavioral response to LEV. Mutations in genes that encode GAR-3, $G\alpha_q$ and their downstream effector PLCβ, result in males that do not protract their spicules in LEV as well as the wild type. The $G\alpha_q$/PLCβ signaling has been shown in the *C. elegans* and other organisms to regulate secretion of neurotransmitter from the presynaptic cells. I found that though $G\alpha_q$ is broadly expressed in every cell of the worm, GAR-3 is only expressed in a small set of cells, including the SPC, PCB and PCC neurons that regulate the male mating. It is also expressed in the spicule protractor muscles, however, at a much lower level. Therefore, the major site of action of GAR-3

signaling is likely to be the spicule-related neurons, and it facilitates the ionotropic AChRs singling probably through upregulating neurotransmitter release from these cells.

The role of the GAR-3 signaling in regulating synaptic transmission is also suggested by results of the following experiments. When I applied a GAR-3-specific agonist, oxo M, to wild-type males, the hyperactivated GAR-3 signaling caused these males to protract their spicules. Oxo M-induced spicule protraction requires the SPC and PCB neurons, cholinergic synaptic transmission and the L-AChR on the spicule protractor muscles. This indicates that the neuronal GAR-3/$G\alpha_q$ signaling is hyperactivated by oxo M to induce spicule protraction, probably through upregulating ACh release from these neurons. Consistently, restoring the *gar-3* gene back to spicule-related neurons fully rescued the oxo M sensitivity in the *gar-3(0)* mutant males.

GAR-3 is also expressed in the spicule protractors at a very low level. When the *gar-3* gene was overexpressed in the male sex muscles it restored the *gar-3(0)* mutant males' ability to protract their spicules in oxo M and LEV. This suggests that when overexpressed, the GAR-3 mAChR on the sex muscles can hyperactivate its downstream effectors in the muscle cells to cause muscle contraction. These effectors, though not directly causing spicule protraction under physiological conditions, might facilitate muscle contraction in parallel with the neuronal GAR-3/$G\alpha_q$ signaling.

The GAR-3 mAChR is not sensitive to LEV. Interestingly, active GAR-3 receptor is required for LEV to induce spicule protraction in virgin males at the wild-type efficiency. This indicates that GAR-3 is activated in animals that are not exposed to agonists or hermaphrodite. This raises a question: in the virgin males I essayed, when do

postsynaptic cells in the spicule circuit become exposed to secreted acetylcholine? I favor the idea that before and between periods of copulation, the neurons in the spicule circuit release small amounts of neurotransmitters that are partially regulated by the GAR-3(mAChR)/$G\alpha_q$ pathway. Therefore the postsynaptic or presynaptic GAR-3 is then activated by the spontaneously released ACh (Fig. 18).

Spontaneous neurotransmitter release has been observed at the neuromuscular junctions in other organisms, such as frogs, flies, and rodents, and was first observed in the frog neuromuscular junction (Fatt and Katz, 1952). In absence of stimulation, pre-synaptic cells spontaneously secrete ACh at a low frequency to activate ACh receptors on postsynaptic cells, causing miniature membrane depolarization. In *C. elegans*, the miniature postsynaptic potential has been measured at the body wall muscle neuromuscular junction. $G\alpha_q$ signaling in the ventral cord motor neurons were found to contribute to the miniature postsynaptic current (Richmond and Jorgensen, 1999). Here I hypothesize that this general phenomenon of background level of ACh release also occurs in the spicule circuit, and it is partly regulated by the GAR-3/$G\alpha_q$ signaling. Others have previously shown that spontaneous neurotransmitter release can regulate the clustering of receptors on postsynaptic cells (Saitoe et al., 2001). In the spicule protraction circuit, a similar mechanism might be occurring. GAR-3(mAChR)/$G\alpha_q$ might define the cholinergic sensitivity of the postsynaptic cells either by regulating the number or clustering of ionotropic AChRs, activating a facilitator of the nAChR signaling, or by inhibiting mechanisms that reduce the cell excitability.

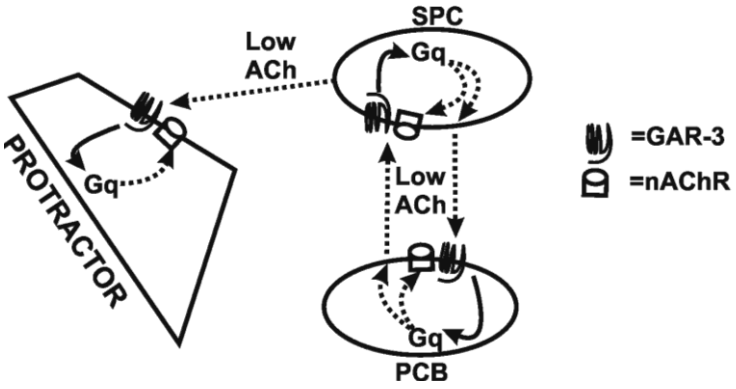

Figure 18. Model of GAR-3-mediated cell interaction.
Prior to mating, activated GAR-3(mAChR)/Gα_q on the SPC and PCB neurons promotes low levels of acetylcholine release. The low level release of acetylcholine also indirectly results in an increased sensitivity of postsynaptic nAChR signaling potential on the spicule protractor muscle cells, and possibly also in the SPC and PCB neurons.

The *gar-3(0)* mutant males that lack the mAChR/Gα_q-mediated signaling have mild behavioral defect when their tails locate the hermaphrodite vulva (Fig. 8). Their phenotype can either be explained by lack of efficiency to insert spicules, or by poor ability to maintain their tail position at the vulva. I hypothesized the cholinergic synaptic transmission between the spicule-related neurons and their postsynaptic cells was one of the sources of signals that attenuate male backward locomotion during vulva searching. Therefore, the *gar-3(0)* mutant males mating defect might be attributable to decreased ACh secretion from these neurons (Fig. 18). In Chapter IV, I demonstrate that the cholinergic synaptic transmission between the p.c.s. neurons and the oblique muscles mediated by ionotropic AChRs is important for males to maintain their tail position at

the vulva. Therefore, it is likely that the GAR-3 signaling facilitate the male mating at least partially via enhancing the ionotropic AChRs-mediated synaptic transmission at this specific site.

Future directions

The goal of this dissertation is to understand the functional structure of the *C. elegans* male copulation circuit, and how featured molecules shape the distinct physiology of the circuit components in order to give rise to a coordinated motor behavior that is important for reproduction. My study demonstrated that the *C. elegans* male utilizes a compact circuit to achieve vulval contact and spicule insertion during mating. After initial recognition of the vulva, the p.c.s. sensory-motor neurons are continuously stimulated by the vulval signal and innervate the oblique muscles via cholinergic synaptic transmission. The oblique muscle contraction then facilitates the contact between the male tail and the hermaphrodite's vulva. A modulatory signaling mediated by the GAR-3/mAChR and its downstream effector $G\alpha_q$ might be used to enhance synaptic transmission between the p.c.s. neurons and these muscles, therefore positively regulating the vulval contact behavior. Activities of the oblique muscle as well as the gubernacular muscles can be relayed to the spicule protractor muscles to induce Ca^{2+} currents and promote rhythmic spicule thrusts at the vulva. The signal molecules are considered to be conducted from the oblique-gubernacular muscle group instantaneously to the protractors through gap junctions to induce Ca^{2+} currents in the

protractors. For the protractors to switch from rhythmic shallow contraction to tonic full contraction, activation of the SPC sensory-motor neurons is required in addition to the already-activated p.c.s. neurons. Additionally, the hook sensillum neurons inhibit the spicule activity until they sense the vulva signal. Therefore, multiple sensory inputs are integrated for the circuit to decide when is proper to fully insert the spicules.

Understanding this basic operational logic of the copulation circuit enable us to study functional molecules that are recruited by the circuit to generate different motor outputs, as well as modulatory mechanisms that fine-tune the circuit activity under different internal and environmental conditions. The following are some questions that will need to be addressed to further our understanding of cellular and molecular regulation of complex animal behavior: 1) how are different functional gap junctions utilized by the copulation circuit to mediate its physiological function; 2) what neural mechanism is mediating the inhibitory effect of the hook sensillum.

How are different functional gap junctions utilized by the copulation circuit to mediate its physiological function?

In adult worms, gap junction structures broadly exist in the male-specific nervous system as well as the hermaphrodite nervous system, and a large number of neurons in *C. elegans* are connected to other neurons or muscles via gap junctions (Male Wiring Project)(Sulston et al., 1980; White et al., 1986; Altun et al., 2009). Although gap junctions have been vastly considered as passive channels that allow molecules of

certain sizes to pass (such as ions, signal molecules), they seem not to simply connect two cells and equalize their membrane potential in *C. elegans*, otherwise neurons and muscles of the *C. elegans* would be activated simultaneously given the extensive gap junction structures that connect them. It is possible that gap junctions formed by different subunits (called connexins) have distinct properties to allow different molecules to pass, so different pairs of excitatory cells connected by different gap junctions are electrically coupled differentially; or the conductance varies under various physiological conditions, therefore the exchange of small molecules only occurs between a specific pair of cells in a particular behavioral context.

C. elegans has long been used as a model organism to study neural regulation of behavior; however, very little has been studied to understand the roles of different gap junctions in regulating behavior, compared to the effort that has been put to understand chemical synapse or extra-synaptic signal transduction. Among 25 genes that encode the *C. elegans* innexins (the invertebrate version of connexins), only a few have been assigned a function in regulating behavior. The INX-16 innexins on the intestine cells couple these cells, so Ca^{2+} waves can be passed through the intestine to promote defecation (Peters et al., 2007). INX-6 and EAT-5 innexins are involved in electrical coupling of the pharyngeal muscles to regulate the feeding behavior (Starich et al., 1996; Li et al., 2003). The UNC-9 innexin is used to electrically couple ventral cord neurons to facilitate wild-type locomotion, and it probably plays a similar role in coupling body wall muscles in regulating the same behavior (Liu et al., 2006; Chen et al., 2007).

My work suggests that certain signal molecule can be passed from the gubernacular-oblique muscle group to the protractor-anal depressor muscle group, probably through gap junctions, to induce regenerated Ca^{2+} currents. These gap junctions thus play important roles in regulating the bistable states of the protract muscle contraction and also in synchronizing the vulval contact behavior and rhythmic spicule thrusts. More interestingly, the two groups of muscles connected by these junctions display different contractile states when the channels are open and allow molecules to pass through. It would be interesting to determine what property of these gap junctions gives rise to this phenomenon. What we will learn from this then is likely to be applied to a more general population of electrical junctions in *C. elegans*.

A gap junction is formed by two hemichannels from each of the cells that are connected by the junction, and each hemichannels contains six subunits, called connexins in vertebrates and innexins in invertebrates. The *C. elegans* genome encodes 25 innexin proteins. Analyzing the expression patterns of all 25 innexin genes in *C. elegans* males is the first step to determine which types of gap junctions are used in the copulation circuit. The transcriptional innexin-GFP fusion markers have been made to survey the expression patterns of all possible gap junction types in *C. elegans* hermaphrodites (Altun et al., 2009). Examining a small portion of these markers in males has shown that both *unc-14* and *unc-9* are expressed in the spicule circuit. Conducting a thorough survey of all innexins should reveal more players in the circuit. In addition, innexin gene genomic sequence-GFP fusion constructs can be further made to provide a more accurate innexin expression map.

My result suggests that the UNC-9 innexin is used to form the gap junctions between the gubernacular-oblique muscles and the protractor-anal depressor muscles. However, as the *unc-9(lf)* males still displayed residual light-induced spicule thrusts, there must be other innexin/innexins utilized by the spicule circuit to form gap junctions. Though RNAi of several other innexin genes did not affect the light-induced spicule thrusts, it could be a consequence of inefficiency of RNAi. The knock-out alleles have been made for large number of genes in *C. elegans*, including many innexin genes, and requests can be made to the *C. elegans* Gene Knockout Consortium to knock out specific genes. Therefore, by carefully checking phenotypes of these innexin knockout mutants, one would determine all innexins used in the spicule circuit.

Gap junctions are formed by hemichannels from both cells that are coupled. The innexin genes that are expressed on both groups of sex muscles are candidates to form the functional electrical junctions that contribute to the rhythmic spicule thrusts. Nonetheless, it has been suggested recently that in *C. elegans,* heterotypic gap junction channels might be formed by two homomeric hemichannels that contains different sets of innexins (Starich et al., 2009). Therefore, any innexins expressed on either group of muscles should be tested for their roles in mediating rhythmic spicule thrusts. By studying phenotypes of mutants of interest, in conjunction with cell-specific rescuing of individual innexin gene in the corresponding mutant males, the innexin/innexins that form our favorite gap junctions could be determined.

Gap junction structures also exist among other components of the male copulation circuit. For example, the SPC neurons and the p.c.s. neurons are connected to

one another; the male sex muscles are also connected via gap junctions (Fig. 11). Given that these cells have distinct functions during male mating and are activated under different circumstances, this raises questions such as, what signals are passed through these gap junctions and what are their roles in regulating the mating behavior? Since we now have a better understanding of how different cellular components interact to give rise to distinct behavioral patterns during mating, and we can measure cell activity via monitoring the Ca^{2+} current in behaving males, it is possible to analyze defective phenotypes of mutant males that have defective gap junctions, even when the defects are not obvious. This study will further our understanding of how various types of gap junctions mediate different cell-cell interactions, and how these interactions shape the patterns of the behavior.

What neurotransmitter and its receptors are mediating the inhibitory effect of the hook sensillum?

In Chapter V, I've shown that removal of the hook sensillum structure enhanced light-induced tonic spicule protraction. In this assay, the males were separated from hermaphrodites and the SPC and p.c.s. neurons were stimulated by light-activated ChR2. This indicates that the hook neurons HOA and/or HOB are active in absence of the vulval signal and are suppressing the spicule activity, and that this suppression is probably released when the male locates the vulva. Consistent with this, a previous study has shown, and it was also observed by me, that when ablated with the hook neurons, the

adult males occasionally display spicule prodding behavior at random places along the hermaphrodite cuticle during mating (Garcia et al., 2001). This suggests that the activated hook neurons not only suppress prolonged spicule protraction but also inhibit rhythmic spicule thrusts. Nonetheless, it is not known through what neural mechanism the hook neurons exert their suppression to the spicules.

HOA and HOB do not have any chemical synapse or electrical junction to the spicule protractors or any other male sex muscles. Instead, the HOB neuron has intensive chemical synapses and gap junctions to the CP5 and CP6 male-specific ventral-cord motor neurons, which innervate the spicule protractor muscles and the oblique muscles. In addition, both HOA and HOB have large numbers of chemical synapses and gap junctions to the spicule-related neurons (SPC and p.c.s. neurons) (Male Wiring Project). I hypothesize that the inhibitory signaling can be relayed from the hook neurons through either the ventral-cord neurons or the spicule-related neurons to suppress spicule activity.

To ask if the CP5 and CP6 neurons act as interneurons to transmit the inhibitory signal to the protractors, these neurons could be laser-ablated in males that express ChR2 in the SPC neurons and the p.c.s. neurons (by expressing the P*unc-103F:ChR2::YFP* construct described in Chapter V). If ablating these neurons results in males that display higher incidence of light-induced spicule protraction, similarly to those that had hook sensillum ablated (Chapter V), it then suggests that the hook sensillum neurons suppress spicule activity via these motor neurons. It remains ambiguous which neurotransmitters are secreted by the hook sensillum neurons, and they could be excitatory or inhibitory.

The CP5 and CP6 have been suggested in a previous study to release serotonin, and therefore activate receptors on the male-specific diagonal muscles at the male tail to facilitate ventral turning of the male tail during mating (Loer and Kenyon, 1993). Data from our lab (Correa unpublished data) shows that serotonin can inhibit ACh agonist-induced spicule protraction in wild-type males. These observations indicate that CP5 and CP6 might negatively regulate spicule activity, and that they are activated by the hook neurons in absence of the vulval signal. Four serotonin receptors have been described and cloned in *C. elegans*, and five more putative receptors that are highly homologous to human 5-HT receptors have been identified (Olde and McCombie, 1997; Hamdan et al., 1999; Ranganathan et al., 2000; Hobson et al., 2003; Carre-Pierrat et al., 2006; Hobson et al., 2006). By analyzing their expression pattern in the male circuit and mutant phenotypes, the receptors that are mediate the inhibitory synaptic transmission between the CP motor neurons and the protractor muscles could be determined.

The HOA and HOB neurons might also suppress spicule protraction via down-regulating activities of the spicule-related neurons. In Chapter V, I showed that when I preselected males that displayed very high expression of P*unc-103F:ChR2::YFP,* these males displayed higher incidence of light-induced spicule protraction compared to the un-biased males. This suggests that by altering the abundance of ChR2 on the cell membrane, the spicule-related neurons can be depolarized at different levels. In *C. elegans,* it seems that electrical signals can either be propagated passively or regeneratively in an all-or-none fashion, depending on the neuron types. Due to the lack of voltage-gated Na^+ channel, *C. elegans* neurons are thought to not be able to generate

classic action potentials (Bargmann, 1998), and lack of regenerative action potential has been indicated in both sensory neuron type and interneuron type(Goodman et al., 1998). Nonetheless, a recent study has shown that regenerative plateau potentials can be recorded in a motor neuron (Mellem et al., 2008; Lockery and Goodman, 2009). In addition, the *C. elegans* ventral cord motor neurons have been shown to release neurotransmitter in a graded fashion (more neurotransmitter released when neurons are more depolarized)(Liu et al., 2009). Therefore, it is possible that the suppression from the hook neurons prevents the spicule-related neurons from being more depolarized, and as a result these neurons release less ACh as a result. To find out if hook neurons directly suppress activities of the spicule-related neurons, ChR2 could be exclusively expressed in the hook neurons, and the Ca^{2+} current indicator G-CaMP could be expressed in the SPC and the p.c.s. neurons to ask whether light-activated hook neurons would suppress activities of the spicule-related neurons. As hook neurons have chemical synapses and electrical junctions to the spicule-related neurons, they could exert their inhibitory function via both types of connections.

Conclusion

C. elegans has been used as a model organism for genetic studies of nervous system function in regulating animal behavior. Mutations that affect various aspects of animal behavior can be easily obtained from mutagenesis screening, and genes that are affected can be determined conveniently. This allows many discoveries of novel

signaling pathways that are important for regulating behaviors, and also provides an *in vivo* system to test molecular mechanisms that have been suggested in cell culture studies. To give rise to complex motor patterns, a behavioral circuit needs to integrate multiple sensory inputs, transform them into simultaneous or sequential contractions in different muscle types, and continue or cease a certain motor output according to real-time monitoring of environmental or internal conditions. Understand the basic operational logic of the behavioral circuit and its functional connections will help us to design mutagenesis screens aimed to understand more specific aspects of the behavior. It will also expedite our understanding of different molecules in regulating behaviors, and will provide frame work to study how environmental and internal conditions exert their modulation to behavior via diverse molecular signaling pathways.

The *C. elegans* male spicule insertion behavior can be viewed as a simple motor behavior. However, evidence has shown that it is under strict regulation of multiple positive/negative cellular and molecular mechanisms. These mechanisms are probably selected through evolution and are used to ensure the male functions properly under various conditions and throughout his life history. Obtaining a thorough understanding of how *C. elegans* males sire their progeny will eventually help us to understand how animal behaviors are shaped and evolved, and this understanding will shed a light on where the human brain is evolved from.

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APPENDIX A

SUPPLEMENTARY FIGURES AND TABLES

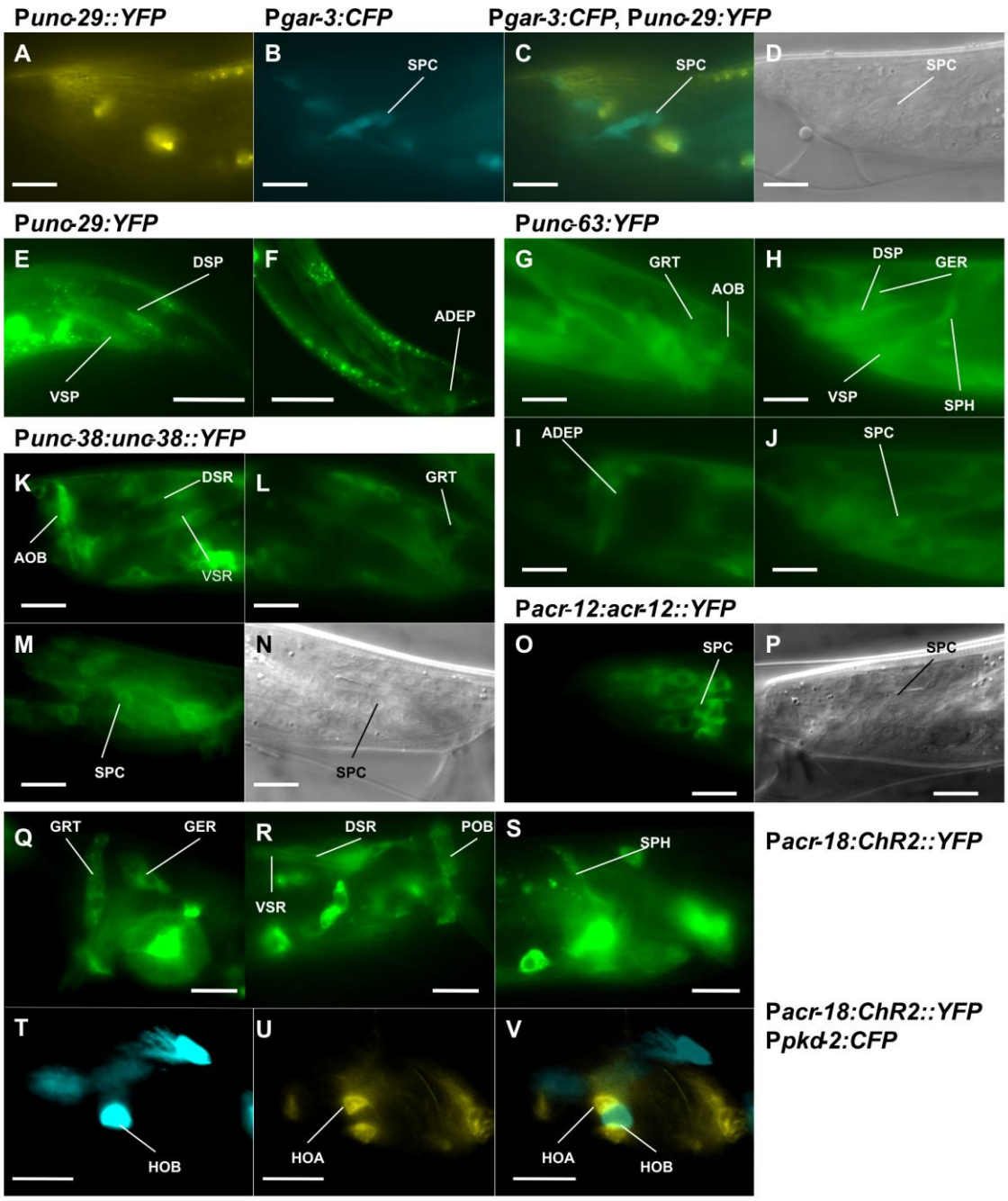

Figure A-1. Expression of nAChR genes in the male tail.
Muscle abbreviations: DSP: dorsal spicule protractor; VSP: ventral spicule protractor; DSR: dorsal spicule retractor; VSR: ventral spicule retractor; ADEP: anal depressor; GER: gubernacular erector; GRT: gubernacular retractor; AOB: anterior oblique; POB: posterior oblique; SPH: sphincter;
A-D. Expression of P*unc-29:YFP* (yellow) and P*gar-3:CFP* (cyan) imaged from the tail of a same L4 larval male. The two images are merged to show that *unc-29* is not expressed in the SPC neurons where *gar-3* is expressing (C). The normarscky image of the tail is also shown (D).
E, F. Expression of P*unc-29:YFP* in the male tail.
G-J. Expression of P*unc-63:YFP* in the male tail.
K-N. Expression of P*unc-38:unc-38::YFP* in the male tail.
O, P. Expression of P*acr-12:acr-12::YFP* in the male tail.
Q-S. Expression of P*acr-18:ChR2::YFP* in the male tail.
T-V. Expression of P*acr-18:ChR2::YFP* (yellow) and P*pkd-2:CFP* (cyan) imaged from the tail of a same adult male. The two images are merged to show that *acr-18* is expressed in the HOA neuron that is next to the HOB neuron where *gar-3* is expressing (V).

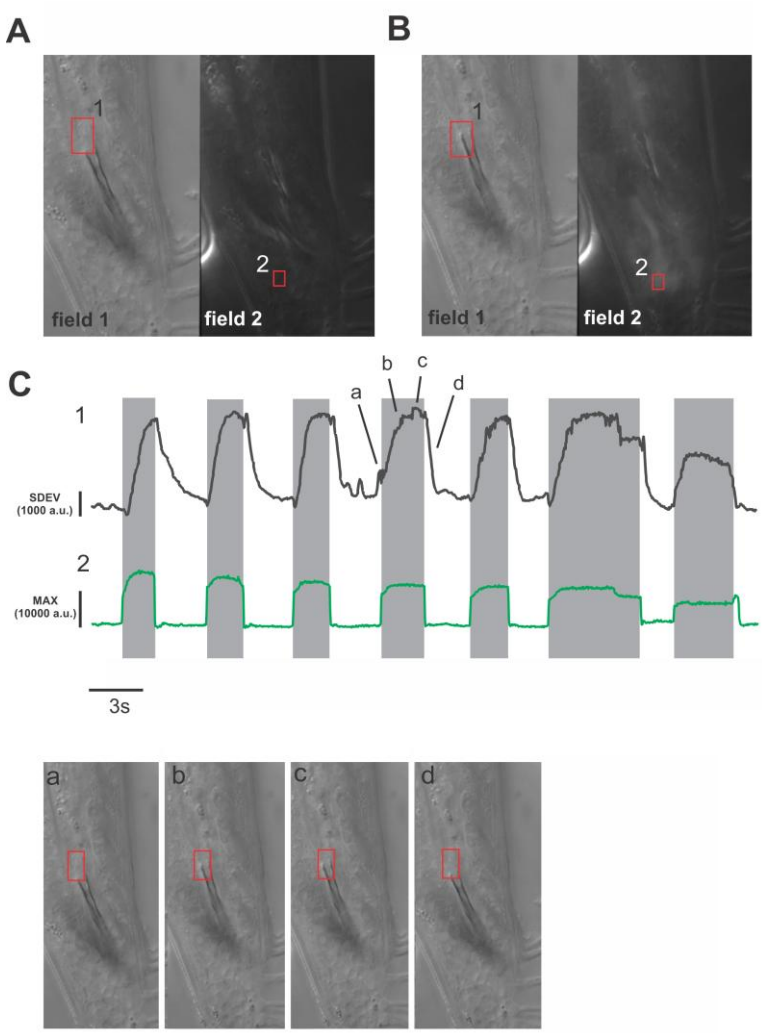

Figure A-2. Brief blue light pulses induce rapid repetitive spicule thrusts (method). Red boxes depict the region of interest (ROI) and are numbered. ROI 1 is used to detect the spicule displacement, and ROI 2 is to indicate the timing of blue light stimulation.
A. A representative frame taken when the blue light was off. The pixel intensity in ROI 2 was low.
B. A representative frame taken when the blue light was on. The pixel intensity in ROI 2 was high.
C. The standard deviation of the pixel intensity within ROI 1 (SDEV) was measured and plotted as a function of time (black trace). Changes in the SDEV indicate displacement of the spicule during and between the periods of light stimulation. The green trace presents the maximal pixel intensity in ROI 2 as a function of time. The increases indicate pulses of the blue light (see Materials and Methods). The grey regions also indicate the time periods of light stimulation. Panel a, b, c and d are representative frames of field 1 taken during and between blue light pulses, and changes in refraction of the ROI 1 region are indicated. The time points when these frames were taken are indicated in the ROI 1 SDEV trace (black). a.u. arbitrary units.

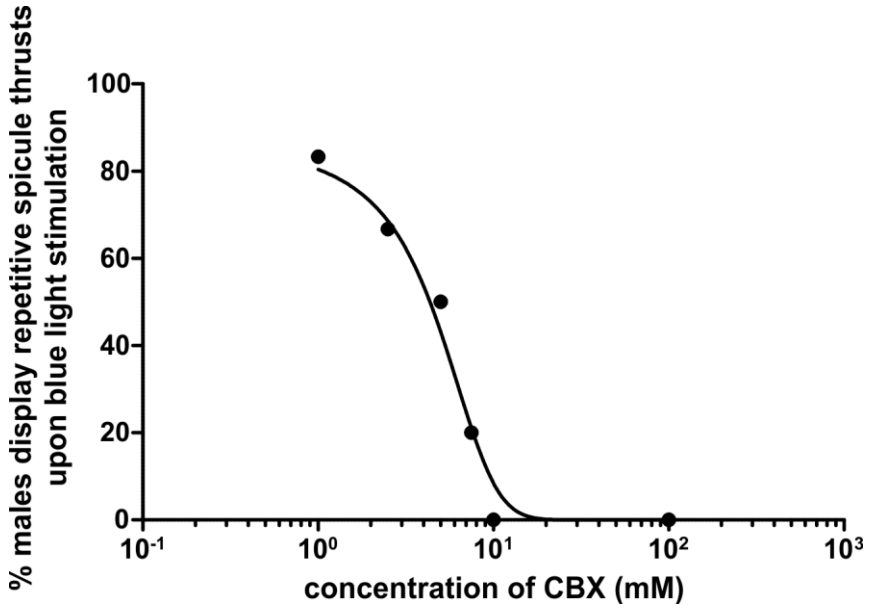

Figure A-3. Carbenoxolone inhibition of blue light-induced repetitive spicule thrusts. CBX was injected at the concentration of 1 mM, 2.5 mM, 5 mM, 7.5 mM and 10 mM, to differentially suppress light-induced repetitive spicule thrusts in males that express P*acr-18:ChR2::YFP,* P*unc-103E:G-CaMP* and P*unc-103E:DsRed*. ~10 males were assayed at each concentration. The X-axis plots concentration of the CBX, and the Y-axis plots the percentage of males that still displayed light-induced spicule thrusts. A log(agonist) vs. Normalized response-variable slope curve is fit to the data to estimate the minimal concentration of CBX that can inhibit light-induced spicule thrusts in the majority of the males.

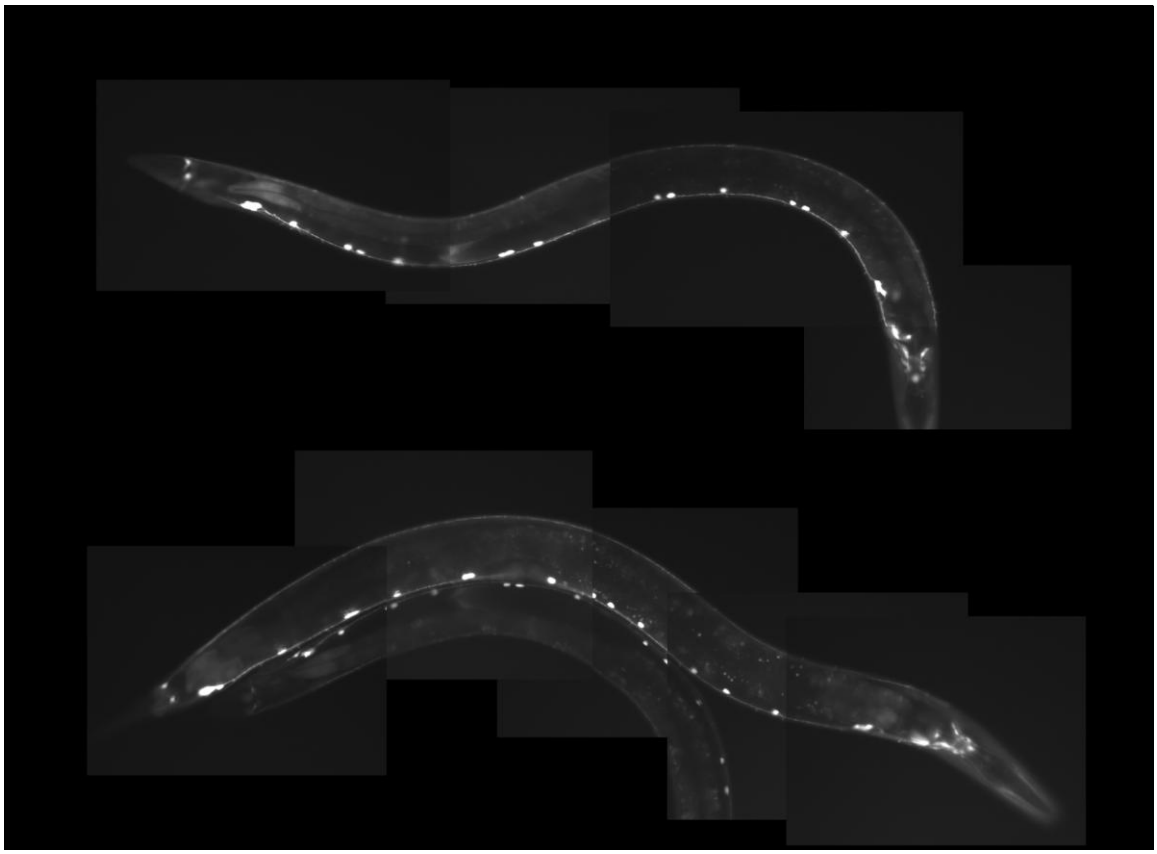

Figure A-4. *gar-3* expression patterns under promoter P*gar-3B* in the male and hermaphrodite.
The male is in the upper panel and the hermaphrodite is in the lower panel.
In cells that are common between male and hermaphrodite, the *gar-3* expression pattern is similar. However, in males, it is expressed in many male-specific muscles and neurons (not shown here), and in hermaphrodites, it is expressed in the hermaphrodite-specific vulva muscles.

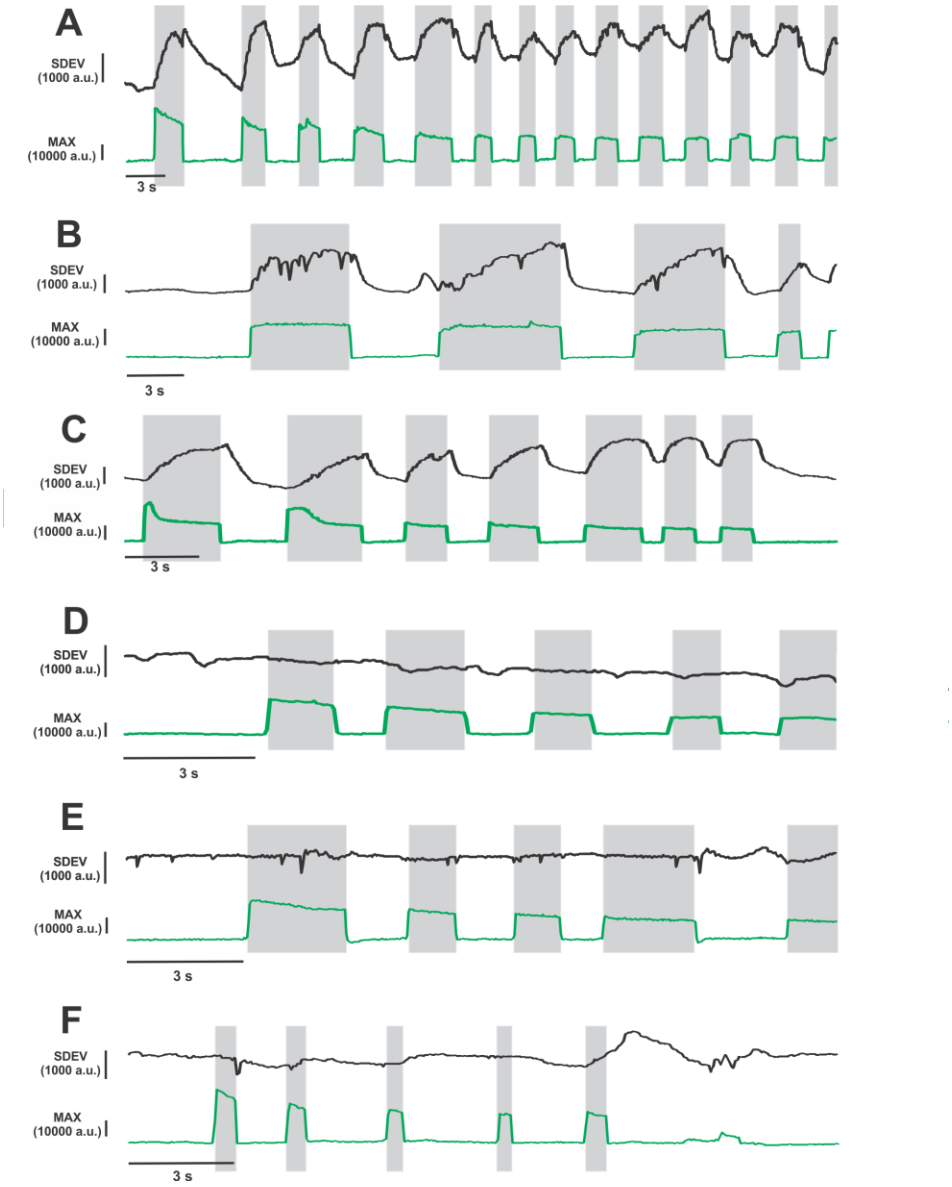

Figure A-5. Brief blue light pulses induce rapid repetitive spicule thrusts (traces of individual males).

Traces represent responses of individual males to blue light pulses. The method used is described in Figure A-2. Black traces indicate displacement of the spicules during and between brief blue light pulses. The green traces indicate pulses of the blue light. The grey regions also indicate the time periods of light stimulation. (A-C) Three males that expressed active P*acr-18:ChR2::YFP*, in the presence of all-*trans* retinal. (D-F) Three males that expressed inactive P*acr-18:ChR2::YFP*, in the absence of all-*trans* retinal.

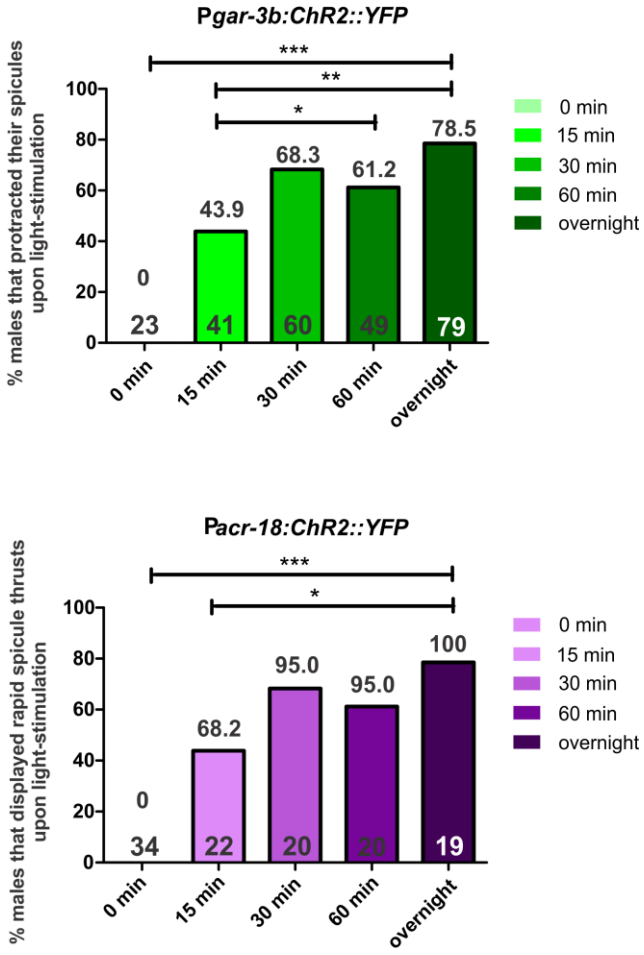

Figure A-6. Correlation between the period of incubation on retinal and the efficiency of light-induced behavior.
To minimize possible change in the G-CaMP/DeRed intensity ratio during incubation with retinal, I determined the minimal period of incubation that is sufficient to elicit ChR2-induced behavior. I used males that expressed P*gar-3b:ChR2::YFP* and males that expressed P*acr-18:ChR2::YFP*. I allowed these males to be incubated on the retinal-containing plates for 0 min, 15 min, 30 min, 60 min and overnight. I then determined the percentage of the male population that protracted their spicules upon blue light stimulation for the P*gar-3b:ChR2::YFP* males (upper panel), and determined the percentage of the male population that displayed rapid spicule thrusts upon light stimulation for males that expressed P*acr-18:ChR2::YFP* (lower panel). I found in both cases, 30 min of incubation was sufficient to induce ChR2-elicited behavior and the efficiency was not significantly different from overnight incubation. The number of males assayed for each strain is listed within each bar (for 0 min, the number is above the X-axis). The number above the bar refers to the percentage of males displayed light-induced behavior. Asterisk (*) indicates the *p* value <0.05, (**) indicate the *p* value <0.001, (***) indicate the *p* value <0.0001,using the Fisher's exact test.

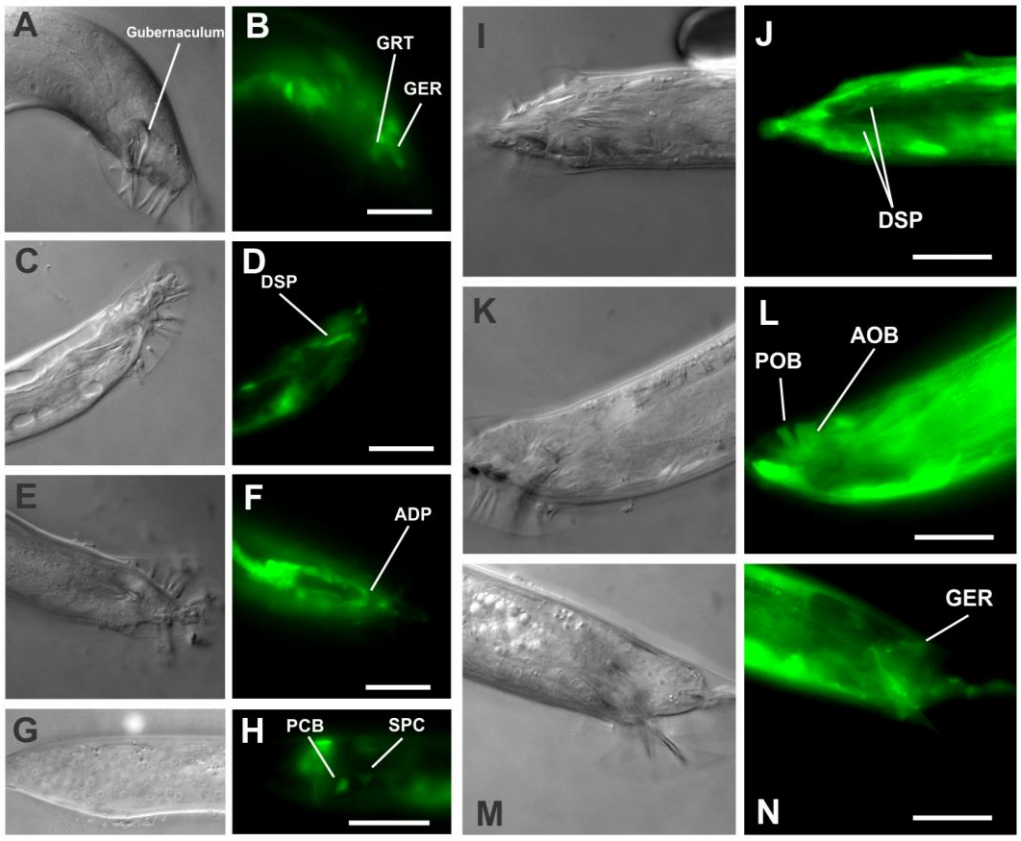

Figure A-7. Male tail expression of *unc-9* and *inx-14*.
Muscle abbreviations: DSP: dorsal spicule protractor; ADP: anal depressor; GER: gubernacular erector; GRT: gubernacular retractor; AOB: anterior oblique; POB: posterior oblique.
A-H. Expression of *unc-9* in the adult male tail (***B***, ***D***, ***F*** and ***H***), and the corresponding DIC images of the male tail (***A***, ***C***, ***E*** and ***G***, respectively).
I-N. Expression of *inx-14* in the adult male tail (***J***, ***L*** and ***N***), and the corresponding DIC images of the male tail (***I***, ***K*** and ***M***, respectively).
All images were taken at 100×. Scale bar, 20 μm. The expression pattern was obtained by using transcriptional GFP fusion constructs (Altun et al., 2009).

Table A-1. Primers used in this study.

Primer name	Primer sequence
ATTB1gar-3Aup	GGGGACAAGTTTGTACAAAAAAGCAGGCTCATAAGCA TCATGAGCAACAT
ATTB2gar-3Adwn	GGGGACCACTTTGTACAAGAAAGCTGGGTCGATTAAT AAATGTGCAGGAG
attb1acr-16	GGGGACAAGTTTGTACAAAAAAGCAGGCTGTGGAGCT GAAGCCTGGGACCGATTGGT
attb2acr-16	GGGGACCACTTTGTACAAGAAAGCTGGGTTACGGACA TGAGAATCAGGGAAAGAAAAGCA
attb1unc-63	GGGGAAGTTTGTACAAAAAAGCAGGCTGCAAGGCTTC TATATACACTACGCATATC
unc-63attb2	GGGGACCACTTTGTACAAGAAAGCTGGGTAACCGTGG TCATTTGGTCCCATTAACCTG
attb1unc-38fus	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGCGGAGG AGTGCTGTTGGGAGCCAT
Attb2unc-38fusnew	GGGGACCACTTTGTACAAGAAAGCTGGGTTGAAACTA ATTGGATTAGCAGATAAATTGG
ATTB1Punc-29	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATCGAG AAGGATGGTTCTCAGTTACACC
ATTB2Punc-29	GGGGACCACTTTGTACAAGAAAGCTGGGTACTGAATG AGAGAATTATA
ATTB2U29lstex	GGGGACCACTTTGTACAAGAAAGCTGGGTAGGGAATA TTGGATGCTGTATCGTATTTCT
ATTB1ACR-18	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCAGAGAA TAATGGACAAAGACTAGGGTCTC
ATTB2acr-18noatg	GGGGACCACTTTGTACAAGAAAGCTGGGATATGGATA AATCAGTATCTGCAAAAGATATC

Table A-1. continued

Primer name	Primer sequence
Func-29	ATGAGGACCAACCGACTATCATGG
unc29R	TCAG GGAATATTGGATGCTGTATCG
Igrgpd3	TCC TGAATTAAAATTAGAAG
Gpd2igr	CAACAGAGTTGTTGATCTCATCTC
Gar3upstrmRv	CATAAGCATCATGAGCAACATCTCCACTTCTCGTGAGC
Dwngar3B	GATTAATAAATGTGCAGGAGGAGTAATAATGGTGTATGT
ATTB1GAR-3strt	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCAGTCCT CTTCGTTGGG GAATGCTGATGATCCTCGAT
ATTB2Gar-3end	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGTTGCGT CGGACATATCCCTGATTCATTGTGGGAC
ATTB1gar-3Bup	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGTTGTTGTC ACAGATTGTCT
ATTB2gar-3Bdwn	GGGGACCACTTTGTACAAGAAAGCTGGGTCCCTCTCGTCT GTGGTGATCC
Gar3xbaF	GGGGTCTAGAATGCAGTCCTCTTCGTTGGGGAATGCTG
Gar3avrR	GGGGCCTAGGGTTGCGTCGGACATATCCCTGATTCATTG

Table A-2. Mutant alleles used in this study.

Gene name	Protein encoded	Allele name	Type of mutation
him-5	unknown; when mutated results in an increased frequency of X chromosome nondisjunction	*e1490*	*lf*
unc-29	L-AChR non-α subunit	*e193*	*lf*
unc-29	L-AChR non-α subunit	*e1072*	*lf*
unc38	L-AChR α subunit	*sy576*	*lf*
egl-30	$G\alpha_q$	*ad805*	*lf*
egl-30	$G\alpha_q$	*tg26*	*gf*
unc73	Trio	*ce362*	*lf*
unc-103	ERG-like K^+ channel	*n1213*	*null*
pha-1	a novel protein that contains a DUF1114 domain of unknown function; when mutated results in temperature sensitive lethal	*e2123*	*lf*
unc-64	syntaxin	*e246*	*lf*
egl-8	PLCβ	*n488*	*lf*
gar-3	mAChR	*gk305*	*null*
acr-16	α7-like nAChR subunit	*ok789*	*null*
lite-1	an ultraviolet light receptor	*ce314*	*lf*
unc-17	synaptic vesicle ACh transporter	*e245*	*lf*
cha-1	choline acetyltransferase	*p1152*	*lf*
unc-9	innexin	*e101*	*lf*

APPENDIX B

MUSCULAR IONOTROPIC ACETYLCHOLINE RECEPTORS THAT ARE REQUIRED FOR ACETYLCHOLINE-INDUCED SPICULE PROTRACTION

It has been showed in a previous study that ACh induces male spicule protraction via activating the LEV-sensitive ionotropic AChR (L-AChR) on the spicule protractor muscles. However, other ionotropic AChRs have also been indicated to mediate this behavior, as the *unc-38(sy576) unc-29(e193)* mutant males that have no functional L-AChR still protracted their spicules in nicotine (NIC), a less specific ionotropic AChRs agonist (Garcia et al., 2001). My data suggests that the ACR-16 containing nAChR is functioning in parallel with the L-AChR in the gubernacular-oblique muscle group to mediate the fast synaptic transmission. As the *acr-16* gene is also expressed in the spicule protractor muscles, it is possible that the ACR-16 nAChR also contribute to ACh-induced spicule protraction.

To determine whether endogenous ACh promote sustained protractor contraction via activating ACR-16, I asked if the *acr-16(ok789)* loss-of-function mutant males had decreased sensitivity to ACh agonists. For NIC-induced spicule protraction, the *acr-16(lf)* mutation by itself did not affect male's response to various concentrations of the drug, similarly to the *unc-38(lf) unc-29(lf)* mutations (Figure B-1)(the original data used to produce this figure are summarized in Table B-1). However, mutant males that lost function of both receptors did not protract their spicules at all, even in very high concentration of NIC (10 mM). This suggests that, the L-AChR and ACR-16 are

interchangeable in mediating NIC-induced spicule protraction; however, when these receptors are depleted from the neuromuscular junctions, there is no receptor can be activated by NIC to promote spicule protraction. This data also indicates that both receptors can be activated by NIC to cause muscle contraction.

It is possible that on the protractor muscles there are receptors that can be stimulated by endogenous ACh but not NIC. To test this possibility, I first asked if another ACh agonist, arecoline (ARE), can still cause the *unc-38(lf) unc-29(lf); acr-16(lf)* triple mutant males to protract their spicules. In previous studies, ARE has been shown to activate a mAChRs-mediated $G\alpha_q$ signaling in the pharynx, and it can also induce spicule protraction through a $G\alpha_q$-independent pathway (Brundage et al., 1996; Garcia et al., 2001). The EC90 concentration of ARE for the wild-type males is 1 mM (Garcia et al., 2001). In contrast, the triple mutant males did not protract spicules at all at this concentration (0%, n=11), and only 7% of these males protracted spicules in 10 mM ARE (n=15). Similarly, these mutant males did not respond to the cholinesterase inhibitor aldicarb either. In 5 mM aldicarb, only 5% of these males fully protracted their spicules (n=40), compared to 100% of the wild type (n=18, $p<0.0001$). This suggests that endogenous ACh promotes sustained spicule protraction in males via activating the L-AChR and ACR-16-containing nAChR on the spicule protractor muscles.

Interestingly, when applied with oxo M at the EC_{90} concentration, 12.7% of the *unc-38(lf) unc-29(lf); acr-16(lf)* males could still protract their spicules (n=205). There are two possible explanations: 1) activation of GAR-3(mAChR) in the spicule protractor muscles can cause muscle contraction autonomously; 2) oxo M activates GAR-3 in the

spicule-related neurons (SPC, PCA and PCB) to promote the activities of these neurons. These neurons then secrete neurotransmitters in addition to ACh, and activate their receptors on the protractors to induce spicule protraction.

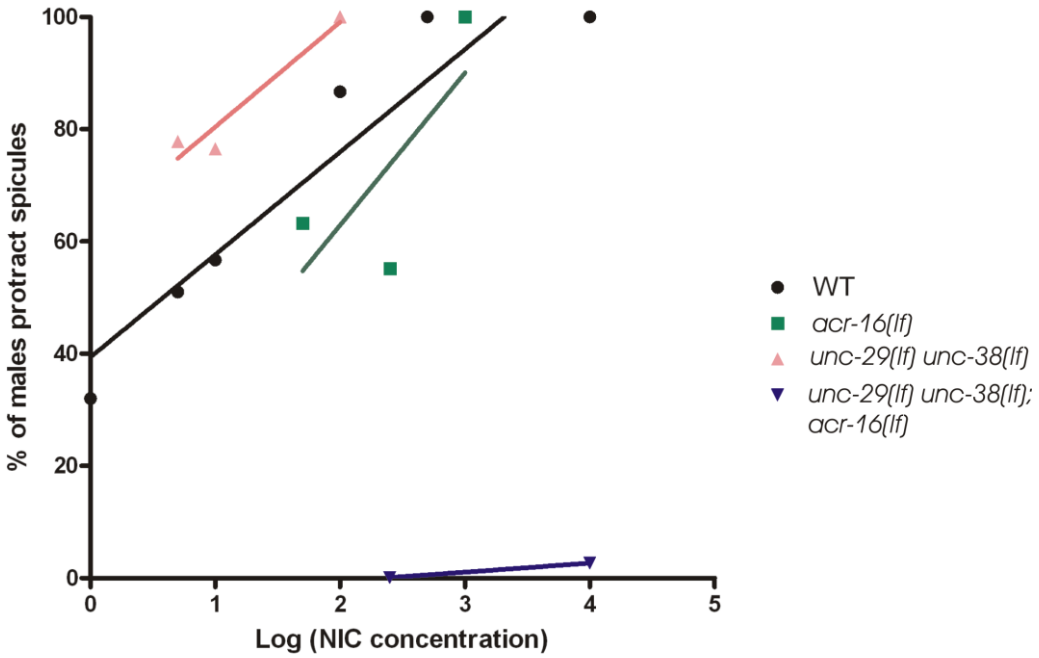

Figure B-1. NIC sensitivity analysis of ionotropic ACh receptor mutants.
For each strain of males, their responses to NIC were tested at multiple concentrations. The Y-axis depicts the percentage of males protracted their spicules in response to the drug. NIC concentrations are in μM.

Table B-1. NIC sensitivity analysis of ionotropic ACh receptor mutants

concentration in µM	*WT*	*acr-16(lf)*	*unc-38(lf) unc-29(lf)*	*unc38(lf) unc-29(lf); acr-16(lf)*
1	32 (50)			
5	51 (28)		77.8 (18)*	
10	56.7 (30)		76.5 (17)*	
50		63.3 (30)		
100	86.7 (30)		100 (19)*	
250		55.2 (29)		0.0 (20)**
500	100 (17)			
1000		100 (17)		
10000	100 (5)			2.7 (37)**

* Comparison was made with WT, and no significant difference can be found using the Fisher's Exact Test.

**$p<0.0001$, compared to WT, Fisher's Exact Test.

APPENDIX C

A MUSCARINIC ACETYLHCOLINE RECEPTOR GAR-2 NEGATIVELY REGULATE THE LEV-INDUCED SPICULE PROTRACTION

My data showed that oxo M can induce spicule protraction in wild-type males, and this agonist-induced behavior is fully suppressed in the *gar-3(lf)* mutant males. This data suggests that oxo M only activates the GAR-3 mAChR to cause protractor contraction. Oxo M has been used as a non-selective mAChRs agonist in the mammalian system, and it can activate both excitatory and inhibitory mAChRs (Freedman et al., 1988; Kaneda et al., 1993; Tayebati et al., 1999; Mistry et al., 2005). It is possible that when applied at a high concentration, this agonist can also activate other mAChRs in *C. elegans*, even though it has been shown that these receptors are not sensitive to low concentration of oxotremorine (Lee et al., 1999; Lee et al., 2000). To test this possibility, I asked if high concentrations of oxo M could suppress LEV-induced spicule protraction in the *gar-3(lf)* mutant males. In 1 μM LEV, 96% of the *gar-3(lf)* males protracted their spicules (n=23), however, in a solution with final concentration of 1 μM LEV and 50 mM oxo M, only 46% of the *gar-3(lf)* males responded (n=28, p=0.0002). This suggests that the negative effect the 50 mM oxo M had on LEV stimulation is independent of GAR-3. When I tested the loss-of-function mutant of another mAChR, GAR-2, in the same assay, I found oxo M no longer suppresses LEV-induced spicule activity. 97% of the *gar-2(ok520)* males responded to 1 μM LEV (n=29), and 94% of these males responded to a mixed solution of 1 μM LEV and 50 mM oxo M (n=34, p=1.0). This

suggests that at the concentration of 50 mM, oxo M can suppress LEV-induced spicule activity via activating GAR-2 mAChRs.

GAR-2 has been shown to couple to Go to act as an inhibitory autoreceptor in the motor neurons (Lee et al., 2000; Bany et al., 2003; Dittman and Kaplan, 2008). My data indicates that a similar signaling pathway might also be used in the spicule circuit to fine tune the circuit activity. Oxo M is probably not a good agonist for GAR-2. Its negative effect could only been detected at a high concentration: 94% of wild-type males protracted spicules in 0.5 μM LEV (n=18), and 94% protracted in a mixed solution with 0.5 μM LEV and 10 mM oxo M (n=17, p=1.000). However, my result suggests a possible scenario that the endogenous GAR-2, once being activated, can negatively modulate the male copulation.

APPENDIX D

THE CP5 AND CP6 NEURONS DO NOT SUPPRESS THE SPICULE ACTIVITY IN THE ABSENCE OF VULVAL SIGNAL

In this dissertation, I have shown that the hook sensillum negatively regulates the ChR2-induced spicule activity. In the absence of vulval signal, the hook sensillum is probably active and suppresses the spicule protractors activity until it eventually senses the vulva and gets inactivated. The hook sensillum neurons, HOA and HOB, do not innervate the protractor muscles directly. Instead, these neurons make intensive chemical synapses and electrical junctions to the SPC neurons, the p.c.s. neurons, and the male-specific ventral cord neurons CP5 and CP6, which directly innervate either the spicule protractor muscles or the gubernacular-oblique muscles (Male Wiring Project).

To determine whether the hook sensillum suppresses spicule activity via the CP5 and CP6 neurons, I laser-ablated these neurons when the males were at the early L4 stage (~37 hrs post-embryo) and asked if the operation would affect the tendency of males to protract their spicules. The CP neurons have been shown to secret serotonin, which activates the diagonal muscles in the male tail to induce tail curling presumably during the “turning behavior” (Loer and Kenyon, 1993). Serotonin can also inhibit animal motor programs such as locomotion and defecation, via activating serotonin-gated chloride channel or G-protein coupled receptors (Segalat et al., 1995; Ranganathan et al., 2000). Therefore, in the absence of vulval signals, the CP neurons might be active to suppress protractor activity, or inactive so they cannot stimulate the protractors. I

found that in wild-type males, ablation of these neurons does not affect males' sensitivity to LEV, which induces spicule protraction via activating receptors on the protractor muscles. In 2 μM LEV, 47.1% of the operated males protracted their spicules (n=34, p=0.435), not significantly different from 58.3% of the intact males (n=24).

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