THE NEURAL AND MOLECULAR MECHANISMS REGULATING MALE LOCOMOTION DURING *Caenorhabditis elegans* MATING BEHAVIOR

A Dissertation

by

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ABSTRACT

In key survival behaviors like predator-prey interactions and mating, animals have to integrate dynamic sensory inputs from a moving target and regulate their motor outputs on moment-to-moment basis. The molecular underpinnings of such goal-oriented behaviors are not well understood because of the genomic and neural system complexities of many animals. Here I take advantage of the anatomical simplicity of the nematode worm *Caenorhabditis elegans* and its amenability to optogenetics to interrogate the neural mechanisms underlying male mating behavior. Male mating is a goal oriented behavioral sequence and serves as a useful paradigm for exploring neural control of sex-specific behaviors, behavioral sequence execution and decision-making. When not engaged in mating the male, like the hermaphrodite, explores his environment with predominantly forward locomotion. However, when the male contacts a potential mate he immediately places his tail against her surface and searches for the vulva, moving backwards. Male-specific sensory rays of the tail are responsible for sensing mate contact, inducing tail apposition and backward movement. Using a combination of cell-specific laser ablation, optogentics and mutant analyses, I show that the male exploits the sex-shared locomotory system to control his mating movement. The rays exert their affect by acting through at least two downstream pathways. One pathway is defined by male-specific interneurons PVY and PVX which activate backward command interneurons AVA(L/R) and shift the directional bias to backward. This AVA activation is mediated by cholinergic receptor subunits ACR-18, ACR-16 and UNC-29,

which is an atypical mode for command interneuron regulation. The second pathway is defined by male-specific interneurons EF1-3. EFs may promote backing by inhibiting sex-shared AVB(L/R) forward command interneurons. Upon vulva detection by the hook sensilla, locomotion ceases by the redundant action of hook neurons HOA and HOB. Surprisingly, PVY/PVX and EFs activity is required for holding the tail at the vulva. Taken together these data suggest that a distributed processing strategy underlies male's accurate, rapid and robust movement control during mating. The male-specific nature of his behavior is due male-specific control of sex-shared circuitry. Conceivably, similar design and processing strategies may underlie the circuitry controlling analogous behaviors in more complex nervous systems.

DEDICATION

This work is dedicated to आई

If not for your sacrifices & strength, it would have been impossible to achieve my dream

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NOMENCLATURE

Ach	Acetylcholine
AchR	Acetylcholine Receptors
ChR2	Channelrhodopsin-2
DIC	Differential Interference Contrast
DRG	Dorsorectal ganglion
EGFP	Enhanced Green Fluorescent Protein
FLP	FMRFamide-related peptides
GECI	Genetically Encoded Calcium Indicator
GFP	Green Fluorescent Protein
NLP	Neuropeptide-like Protein
NpHR	Halorhodopsin
p.c.s.	post-cloacal sensilla
PAG	Pre-anal ganglion
RVG	Retrovesicular ganglion
TRP	Transient Receptor Potential
unc	Uncoordinated
VNC	Ventral Nerve Cord
YFP	Yellow Fluorescent Protein

TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

A rapid and accurate behavioral response is crucial for survival

Survival depends on an organism's ability to respond to its surrounding conditions. The faster and more accurate the response, the higher is the chance of survival. One of the fundamental questions of neuroscience is how do organisms rapidly process the external information to produce an appropriate motor response? The most critical step of information processing is decision-making. Even for the simplest behavior a decision has to be made, whether to continue the ongoing behavior or choose an alternate one. Hence, decision-making is crucial for survival and is conserved across all domains. A single celled bacteria exhibits decision-making by taxing towards an attractant and moving away from a repellant substance (Adler and Tso, 1974). In animals, decision-making becomes much more complicated with higher level of cellular organization (involving specialized tissues like nerves and muscles), which generate complex sensorimotor patterns to produce behavior. Most animals have dedicated neuronal circuits that respond to different external cues. Neuronal circuits sense external stimuli, integrate and process the information changing the muscle activity to perform an action. Furthermore, in goal-directed behaviors such as mating or predator-prey interplay, animals have to respond to moment-to-moment changes in the actions of their targets and respond appropriately, often with split second timing. Hence, sensorimotor

integration, rapid information processing and decision-making become essential features of a neural circuit controlling such behaviors.

The focus of my thesis is to understand how neural circuits execute a goal-directed behavior at the cellular and molecular level. Due to the complexity of higher organisms, I am using the simple nematode *C. elegans* to answer this question. *C. elegans* male mating behavior is an attractive model system to investigate sex-specific behavior, sensorimotor integration, rapid information processing and decision-making. The most important advantage of using *C. elegans* is that we can interrogate the neural circuits at a single cell level in freely-behaving animals and understand the fundamental principles of neural mechanisms.

Neural mechanisms controlling the response to moving targets

One of the extensively studied examples of interactions with moving target is the predator-prey interplay. Animals have to track the position of the predator and rapidly change its own position accordingly to escape. The neural circuits controlling the escape response have to be fast, accurate, robust and flexible. What neural mechanisms and circuit design features confer such properties? Escape circuits are commonly classified into two categories, ones that involve "command" neurons and ones that do not (Kupfermann and Weiss, 1978, 2001). The command system is generally characterized by the presence of giant fibers which are large in diameter and run along the length of the animal, forming direct connections to most of the circuit components (Fotowat et al., 2009, Herberholz and Marquart, 2012). A single spike in the giant fiber is sufficient to

activate the entire escape response (Herberholz and Marquart, 2012). In *Drosophila*, the giant fiber activation leads to motor activity in the mesothoracic legs and wings resulting in jump during escape response (Koto et al., 1981, Wyman et al., 1984). Similarly in crayfish, tail-flip escape response is controlled by three distinct neural circuits. Two of these circuits contain the giant fibers, the lateral giant system and the medial giant system, and execute the escape response. The command neuron centralizes the response in a rapid and stereotypical manner. However the speed of the response often compromises its flexibility. The escape responses which do not involve the command neurons are called "non-giant" responses and are known to be more variable but slow. One such example is the third neural circuit in the crayfish tail-flip escape response which lacks the giant fiber activity (Wine and Krasne, 1982, Wine, 1984). Similar non-giant mediated escape response is seen in locusts (Fotowat and Gabbiani, 2007) and zebrafish (Kohashi and Oda, 2008). The crayfish respond to their natural predators, dragonfly nymph by activating all three neural circuits (Herberholz et al., 2004). However, the non-giant-mediated tail-flips increase subsequent to the failure of giant-mediated tail-flips (Herberholz and Marquart, 2012). This leads us to the question of how do crayfish decide between the giant-mediated and non-giant-mediated responses? The broader implication of this simple example is how neural circuits decide the appropriate response.

Decision making is essential for producing the appropriate behavioral response

Decision making involves evaluating different options and making a choice to perform the appropriate action (Ernst and Paulus, 2005). Failure to make a stable decision can affect a person physically, psychologically, emotionally, socially, and economically (Ernst and Paulus, 2005, Gutnik et al., 2006, Frith and Singer, 2008). However, from a neuroscience perspective decision-making is an important step in getting the right output from a dynamic neural circuit. What happens when these neural circuits malfunction and decision making is impaired? For example, people with damaged pre-frontal cortex or orbitofrontal cortex have a tendency to make high-risk decisions (Ritter et al., 2004, Bechara, 2005, Larquet et al., 2010). When subjects with lesions in these brain regions where tested with Iowa Gambling task, they repeatedly made poor decisions without contemplating the consequences (Bechara, 2001, Ritter et al., 2004). One of the important functions of the prefrontal cortex is to consolidate the internal and external information, thus playing a crucial role in cognitive control of complex behaviors (Miller and Cohen, 2001). The molecular mechanisms underlying the prefrontal cortex functions have been extensively studied (Robbins, 2000, Arnsten, 2009). Also, based on the decision-making studies performed on humans and primates, numerous models have been proposed to explain how decisions are made at the circuit level (Glimcher, 2003, Lee, 2006, Gold and Shadlen, 2007). A further complication is that the brain has to consolidate the novel experiences and compare them with the old ones (retrieving memories) to come to a decision. However, the molecular mechanisms used to integrate the existing and new information during decision-making are not

completely known. Also, the neural mechanisms used to retrieve memories in the prefrontal complex also used to process novel experiences and make a choice?

In humans and primates, decision-making is a complex process which involves multiple neurotransmitter mechanisms. Disorders or conditions with impaired neurotransmission affect sensorimotor integration leading to unstable decisions. Addictive drugs and diseases which damage dopaminergic systems are proposed to impair decision-making (Schultz, 2002, Verdejo-Garcia et al., 2006). Abnormal functioning of the dopaminergic neurons is one of the causative factors in the decline of brain functions in psychiatric conditions like schizophrenia, depression and mania (Wise and Rompre, 1989). Most drugs of abuse like nicotine, cocaine, marijuana and alcohol are known to overstimulate the dopaminergic neurons, leading to heightened euphoric state (Volkow et al., 2011). The dopaminergic system along with the serotonergic system plays a role in impulsivity. More impulsive behavior leads to risky decisions where the individual is highly likely to make uninformed, random decisions rather than correct one (Cools et al., 2011, Dalley and Roiser, 2012). Also, disruptions in cholinergic system through long term cocaine use leads to impaired decisions, primarily due to poor learning and memory (Williams and Adinoff, 2008). The malfunctioning of a single neuronal molecule can alter the internal states of neural circuits which influence decision-making. Our understanding of how neurotransmitters or neuromodulators affect neural circuit functions comes from vertebrate tissue culture studies and invertebrate model system analyses, in which circuits are comparatively simpler to manipulate.

Invertebrate model systems have made seminal contributions to fundamental neuroscience

Experimental studies in a variety of invertebrate model systems have provided significant insight into many aspects of nervous system functions (Sattelle and Buckingham, 2006). In the 1930s, Hodgkin and Huxley recorded the first action potential by placing electrodes in the giant axon of a squid (Hodgkin and Huxley, 1939). In the 1950s, they collaborated with Katz to develop voltage-clamp technique and explained the ionic conductance during the generation of action potential (Hodgkin and Huxley, 1952a, Hodgkin et al., 1952b). Since then, experiments performed on giant fibers of other invertebrates have revealed neural mechanisms involved in rapid responses. Giant fiber caliber and their electrical coupling with sensory and motor neurons play a key role in their speed of neurotransmission (Palikhova et al., 2006, Hartline and Colman, 2007, Yono and Aonuma, 2008). The principle of lateral inhibition was first discovered by Hartline in 1949 by studying photoreceptors in the horseshoe crab, *Limulus*. Eric Kandel discovered the mechanisms for simple forms of learning and memory (habituation, dishabituation and sensitization) by studying the gill-withdrawal reflex in *Aplysia* (Castellucci et al., 1970, Castellucci and Kandel, 1976). The pioneering work of Eve Marder in neuromodulation of somatogastric nervous system of lobsters revealed that the constituents of neural circuits can change in response to the influence of neuromodulators (Eisen and Marder, 1984, Marder, 1984, Marder and Hooper, 1985, Marder, 2011). These general principles of the neural circuit design and processing

strategies in the smaller invertebrate systems provide an insight into how larger, complex vertebrate systems.

In recent years, *Drosophila melanogaster* and *Caenorhabditis elegans* have emerged as powerful model organisms. Their relative anatomical and genetic simplicity, completely sequenced genomes and their amenability to genetic and molecular approaches have made these systems ideal for studying almost any question. Research employing these systems have shed light on the neural and molecular underpinnings of neurodegenerative diseases, substance abuse, memory and learning and sensory-motor integration (reviewed in (Wilson-Sanders, 2011). *Drosophila* and *C. elegans* male mating provide paradigms for exploring many neural processes. These behaviors are innate, goal-oriented, consist of a sequence of stereotyped steps that are guided by mate cues. During mating there are many decision-making points. For example, whether to ignore or pursue a mate, whether to initiate mating or not and so on. The dependency on mate cues to execute the mating behavior provides a model for studying how sex-specific behaviors are neurally controlled. Not much is known about the sex-specific behaviors. For example, are mating sub-behaviors controlled by dedicated sex-specific circuits or by mate cue-specific modulation of the sex-shared neural circuitry?

My work explores decision-making events during locomotion in *C. elegans* male mating behavior. First, I will give a brief overview *Drosophila* male mating behavior which has similar mating pattern. Then I will discuss in detail what is known about *C. elegans* male mating and locomotory behavior.

Decision making in *Drosophila* mating behavior

The male courtship behavior is a sequence of motor outputs which are guided by sensory cues received from the females. On detecting the pheromone signals from the female fly, the male orients himself towards the female and follows her. He taps the female abdomen with his forelegs, extends and vibrates his wings producing a male-specific song which stimulates the female. Based on her prior experience and her assessment of the courting male, the female decides whether to mate or not mate. If the female decides to reject the male she extrudes her ovipositor, kicks and flies away. If the female decides to mate, then she slows down allowing the male to attempt copulation. The male responds to the female acceptance by licking her genitalia and bending his abdomen to attempt copulation (Dickson, 2008, Dauwalder, 2011, Pavlou and Goodwin, 2013).

In flies, two genes *fruitless (fru)* and *doublesex (dsx)* contribute to the development and functioning of circuits involved in the mating behavior. Consequently defects in these genes disrupt courtship behavior (Manoli et al., 2005, Stockinger et al., 2005). By identifying FRU and DSX expressing cells researchers have been able to partially delineate the neural circuitry underlying male mating behavior (Dauwalder, 2011). For example, a subset of FRU-expressing sensory neurons regulates olfaction in males and is responsive to female hormones. Another subset is the gustatory receptors, located on the forelegs of males, are responsible for discriminating between males and females during courtship (Pavlou and Goodwin, 2013). These studies show that a dedicated subset of neurons contributes to sensing and pursuing a mate in *Drosophila* mating behavior.

The next question is where are the sensory inputs integrated and if there are any dedicated decision-making neurons? A cluster of *fru/ dsx* expressing cells, P1 is located in dorsal posterior brain and is known to trigger singing in presence of a female. The silencing of P1 neurons leads to impaired song formation and courtship performance (Kimura et al., 2005, Kohatsu et al., 2011, von Philipsborn et al., 2011). Conversely artificial activation of P1 neurons triggers the male to orient towards and follow any object (Pan et al., 2012). These results suggest that the P1 cluster integrate the olfactory and gustatory inputs and decide on the course of action to court the female (Kohatsu et al., 2011). While some of the cellular components of the courtship circuitry have been identified the molecular mechanisms controlling circuit transmission are largely unknown (Pavlou and Goodwin, 2013).

C. elegans as a model organism

C. elegans is a non-parasitic, soil nematode naturally found in decaying vegetation. A fully grown adult worm is about 1mm in length. The small size makes it convenient to maintain and cultivate worms on small plates. *C. elegans* has two sexes, hermaphrodites and males. Hermaphrodites are self-reproducing with a limited amount of sperm supply to fertilize their eggs. Males can reproduce only by mating with the hermaphrodites. The short generation time of 3-4 days (from egg to fertile adult) makes it easier to work with as compared to the vertebrate model systems which have longer generation times. On hatching, the worm grows through 4 distinct larval stages (L1, L2, L3 and L4) and finally develops into an adult (Brenner, 1974). The somatic cell lineage is invariant

(~1000) and cell bodies occupy stereotyped positions in the body axis (Sulston et al., 1980). These features, together with the worm's transparency make cell identification for the purpose of cell-specific ablation or to target gene expression, feasible.

C. elegans has a simple nervous system with 302 neurons in the hermaphrodite and 383 in males (Sulston and Horvitz, 1977, Sulston et al., 1980, Jarrell et al., 2012). A big advantage in using *C. elegans* is that the complete wiring diagram of the hermaphrodite nervous system and a partial map for male have been determined (White et al., 1986, Hall and Russell, 1991, Jarrell et al., 2012). The lineage together with the wiring diagrams reveal that hermaphrodites and males share 294 neurons in common, two third of which are sexually dimorphic (*i.e.* are present in both sexes but are modified in each sex). Males have 89 male-specific neurons, 85 of which are located in the male tail and are proposed to play a role in mating behavior (Sulston and Horvitz, 1977, Sulston et al., 1980). In addition to neurons, other tissue types exhibit sex-specificity or sexual dimorphism. For example, males have 41 male-specific muscles in the posterior half of the body (Sulston and Horvitz, 1977, Sulston et al., 1980). Also, posterior sex-shared muscles like anal depressor, sphincter and dorsal body wall muscles are dimorphic, presumably to facilitate copulation in males. While minor sex differences exist even in the embryo, most sex-specific cell types are added during L3 and integrated with existing cell types by the end of L4. The most striking difference in male and hermaphrodite development is the tail part. The male tail has majority of the male-specific and sexually dimorphic tissue types and guides the male mating behavior.

C. elegans male mating behavior

Similar to *Drosophila* mating behavior, the *C. elegans* male performs a sequence of motor behaviors driven by presumptive chemosensory and mechanosensory cues from his mate (Liu and Sternberg, 1995, Simon and Sternberg, 2002, Lipton et al., 2004). In absence of specific cues, the male explores his environment moving with a forward directional bias but makes intermittent reversals to change direction (Fig. 1.1A). When the male comes in contact with the hermaphrodite, he stops exploring his environment and starts mating. The decision to initiate mating is affected by the male's recent experience. Adult males deprived of sex are quick to initiate mating whereas males with recent experience go through a refractory period during which mating is not initiated. It is postulated that during this period male mating circuits are reset for the next mating episode (LeBoeuf et al., 2014).

One of the most striking changes observed when the male starts mating is the change in locomotory direction: a switch from forward to backward movement. When the male tail comes in contact with the hermaphrodite (Fig. 1.1B), he apposes his tail, ventral side down, against hermaphrodite surface and moves backward along her length in search of the vulva (the vulva search). The male tail fan straddles the hermaphrodite, bringing the sensory rays and other male-specific sensilla into direct contact with the hermaphrodite cuticle. Collectively, tail apposition and initiation of backward movement are called "contact response." If the male moves along the hermaphrodite (scanning) without encountering the vulva, he makes a tight turn to reach the other side and continues scanning until he finds the vulva. Throughout scanning and turning, the male

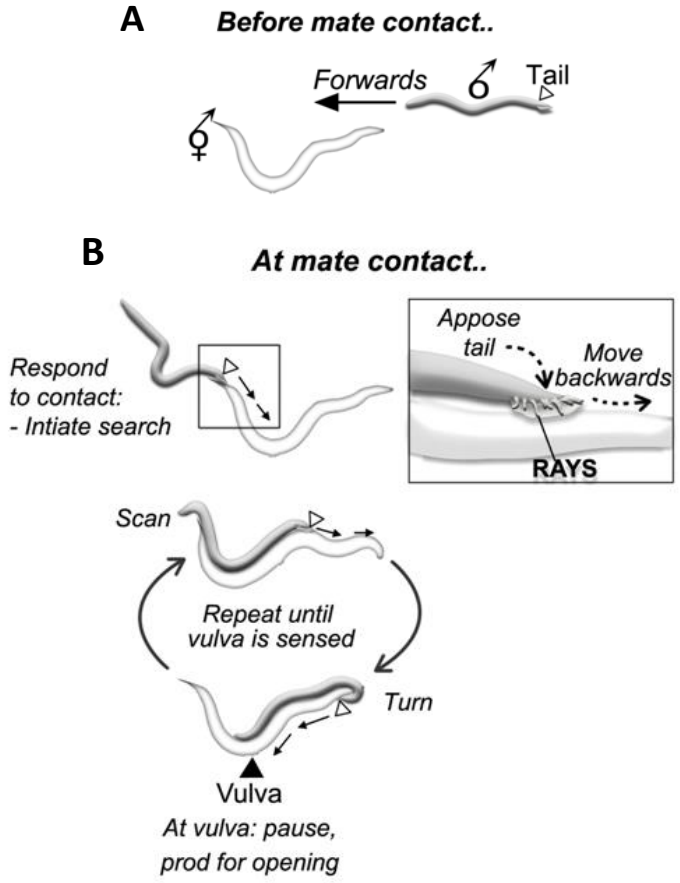

Figure 1.1 *C. elegans* male mating behavior exhibits distinct locomotory patterns.
A, B. Male mating behavior has stereotyped steps.
A. In absence of mate contact, male locomotion has a forward directional bias with occasional backward movement (known as spontaneous locomotion).
B. On mate contact, male initiates the vulva search by stopping abruptly, apposing his tail on the hermaphrodite surface (inset) and changing the directional bias to backward (Koo et al., 2011). This backward directional bias is maintained during scanning, as the male sensory rays drive the contact-based search for the vulva. On vulva detection male stops the backward movement and restricts his locomotory activities to the immediate vulva region. The male finds the precise location of the vulva, starts prodding his spicules against the vulval slit to attempt spicule insertion. The mating behavior is completed on complete spicule insertion and insemination (not shown in the figure).
(taken from Sherlekar et al., 2013)

tail stays in contact with the hermaphrodite cuticle (Fig. 1.1B). On encountering the vulva, male stops but maintains tail apposition approximately over the vulva region. He then starts prodding with his copulatory spicules to precisely locate the vulval slit. Once located, he breaches the vulva, to insert the spicules for insemination.

As in *Drosophila*, the development of sex-specific tissues in *C. elegans* is regulated by *dsx*-related genes but worms lack *fru* orthologues (Raymond et al., 1998, Lints and Emmons, 2002, Mason et al., 2008, Siehr et al., 2011). *dsx*-related mutants have pleiotropic defects, so their analysis has not helped to identify circuits involved in *C. elegans* mating. Most circuit mapping has been guided by the wiring diagram. The connectivity data enables us to predict putative circuits controlling mating behavior. These putative circuits can be tested for relevance by using laser ablations, optogenetics, Ca^{+2} imaging and other molecular tools to understand how sex-specific behaviors are generated. Below I will describe what is known about the neural substrates underlying each step of the male mating behavior.

Contact response and scanning

Male mating behavior is coordinated by the male-specific sensilla of the tail (Fig. 1.2A). The tail sensilla include rays, hook, post-cloacal sensilla (p.c.s.) and spicules that presumably responsive to mate cues. The ray sensilla sense mate contact and induce and guide the vulva search. There are nine, bilateral pairs of sensory rays (numbered 1 to 9; anterior to posterior) embedded in a cuticular fan (Fig. 1.2A). Each ray consists of a structural cell (Rnst; where n is the ray number) and the dendritic ends of two distinct

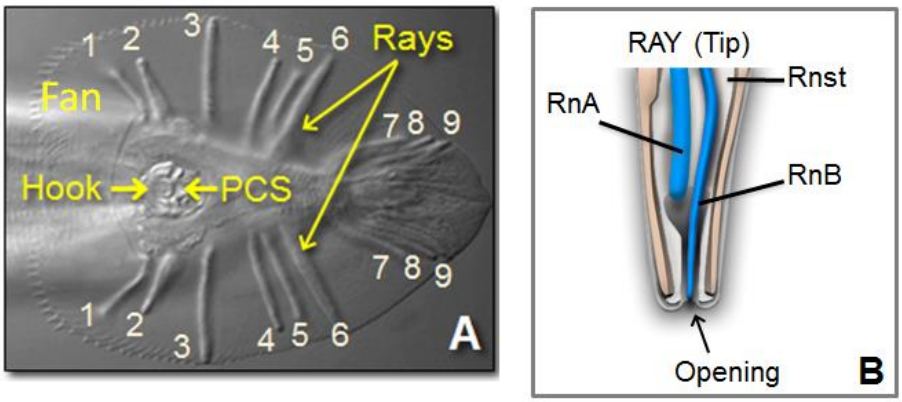

Figure 1.2 Male-specific sensory rays initiate and guide the vulva search.
A. DIC micrograph of male tail (ventral view) showing the nine bilateral pairs of rays (numbered 1-9, anterior to posterior), the hook and post-cloacal sensilla (PCS). Spicules are located inside the male tail (not seen in this picture). Magnification 1000X.
B. A schematic representation one ray ending. Each ray neuron is composed of two sensory neurons RnA and RnB (where n is the ray number). The dendritic process of RnA ends just before the open ray tip. RnB dendrite is exposed to the environment as it extends all the way to the tip of the ray (except for Rn6 ray neurons; they have closed tip). The two ray neurons are supported by structural cell, Rnst. The cell bodies of RnA, RnB and Rnst lie in the tail ganglion (not shown).
(adapted from Koo et al., 2011)

sensory neurons, type A (RnA) and type B (RnB) (Fig. 1.2B). Except for ray 6, the ray tips are open and the dendritic endings of type B ray neurons are exposed to the environment. The dendritic endings of type A ray neurons stop short of the ray tip opening. Rays 1, 5, 7 open on the dorsal side of the fan whereas rays 2, 4, 8 open ventrally and rays 3 and 9 open at the fan margin (Sulston and Horvitz, 1977, Sulston et al., 1980). The sensory modalities and receptors mediating ray neuron response are remains an open question. The ray neurons express TRP channel genes, *pkd-2* and *trp-4*; and *pkd-2* mutants are defective in contact response (Barr and Sternberg, 1999, Li et al.,

2006, Koo et al., 2011). However, experiments demonstrating these as the sensory receptor molecules have yet to be performed.

Males lacking all functional rays are unable to initiate or execute the vulva search (Liu and Sternberg, 1995, Koo et al., 2011). Elimination of specific sub-sets of rays compromises, but does not eliminate the behavior (Koo et al., 2011). Ca^{+2} imaging of ray neuron activity during mating reveal that both type A and type B neurons are active throughout mating behavior. With initial contact, ray neurons exhibit rebound depolarization; the rationale for which is unknown. Following this event, ray neurons like many *C. elegans* neurons exhibit graded activity. The activity increases or decreases depending up on the motor output (R. Lints unpublished; Sherlekar and Lints, 2014). What is the specific contribution of type A and type B ray neurons? The ablation studies done in our lab reveal A-neuron ablated males have impaired posture control and movement, making them inefficient in maintaining contact while turning and scanning. The B-neuron ablated males have difficulty in initiating contact response; however after few failed attempts they can initiate contact and mate with wild type efficiency. Collectively these data suggest that both A- and B-neurons are active throughout mating. A-neuron activity is required for all steps of mating while B-neurons are functionally redundant with A-neurons (Koo et al., 2011). The partial redundancy among the rays and ray neurons explains how ray-controlled behaviors are robust, accurate and reproducible.

The male wiring data shows that sensory rays have both male-specific and sex-shared neurons and muscles as their downstream cellular targets. The ray neurons form massive reciprocal connections among themselves, with other sensory neurons and male-

specific neurons in the pre-anal ganglion (Jarrell et al., 2012). The connectivity and experimental studies suggests that the ray-controlled motor outputs are generated by distributed processing through multiple neural pathways and efferent targets (sensory neurons, interneurons, motor neurons and muscles). For example, activation of B-neurons (using ChR2) in males lacking male-specific muscles impairs but does not block the ventral curling of tail. Similarly, a percentage of males lacking a significant number of male-specific interneurons, motor neurons and muscles can still produce ray-induced posture; however not as robustly. These results also reveals that ray motor outputs depend on both male-specific and sex-shared cells (Whittaker and Sternberg, 2009, Koo et al., 2011). In contrast to our understanding of posture control, the ray-dependent pathways that regulate locomotory changes during mating are completely unknown. A major objective of my thesis is to identify these ray-controlled locomotory pathways.

Turning

During his search for the vulva, if the male reaches the hermaphrodite ends (head and tail regions), he makes a deep ventral bend of his tail to reach the other side. Exposure of worms to endogenous serotonin or dopamine induces a deep ventral curling of male tail similar to the turning posture suggesting that these neurotransmitters are involved in inducing or modulating turning posture (Loer and Kenyon, 1993, Lints and Emmons, 1999, Siehr et al., 2011).

Vulva location

Upon sensing the vulva, the male stops backward movement (Barr and Garcia, 2006). The male-specific hook neurons and p.c.s. neurons are responsible for sensing the general and precise location of the vulva, respectively. The hook sensilla are located anterior to the cloacal opening and consist of two sensory neurons, HOA and HOB. These neurons are structurally similar to the ray neuron types A and B. Like RnB, the dendritic ending of HOB exposed to the environment and like RnA, the HOA process ends inside the sensilla, respectively (Sulston et al., 1980). Based on the location of their endings, HOB is hypothesized to be chemosensory and HOA, mechanosensory (Barr and Garcia, 2006). Males lacking hook neurons, due to their specific ablation, cannot find the general vulva location and perform a slow search in which they prod with their spicules at random location. Although imprecise, this strategy occasionally leads to successful insertion of spicules into the vulva followed by insemination (Liu and Sternberg, 1995). Hook neurons express markers for glutamate, acetylcholine and neuropeptides (B. LeBoeuf and L. R. Garcia, personal communication; Nathoo et al., 2001, Kim and Lee, 2011, LeBoeuf et al., 2014). However, the role of neurotransmitters in hook function has not been extensively explored.

The p.c.s. is located posterior to the cloacal opening and consists of three bilateral pairs of sensory neurons PCA(L/R), PCB(L/R) and PCC(L/R) (Sulston et al., 1980). In the absence of p.c.s. neurons, the males can sense the general vulva location; however they are not able to position themselves precisely over the vulva and consequently fail to insert their spicules (Liu and Sternberg, 1995). Males lacking both

the hook and p.c.s. neurons cannot stop at the vulva and keep scanning, without any slow search or spicule prodding (Liu and Sternberg, 1995, Garcia et al., 2001).

Spicule insertion and insemination

The spicules are a bilateral pair of prong-like structures held within the proctoderm by two retractor muscles (Sulston et al., 1980). Inside each spicule are the dendritic processes of two male-specific sensory neurons SPV(L/R) and SPD(L/R) wrapped in a sheath cell and two socket cells (Sulston et al., 1980). Each spicule is attached to protractor and retractor muscles which facilitate spicule movement. As the muscle name suggests, contraction of protractor muscles causes the spicules to protract out through the cloacal opening and contraction of retractor muscles causes the spicules to retract back into the proctoderm. Upon sensing the vulva, hook and p.c.s. neurons activate the protractor muscles initiating the spicule prodding. The p.c.s neurons are cholinergic (PCB and PCC) (Garcia et al., 2001) and glutamatergic (PCA) (LeBoeuf et al., 2014). Their activation stimulates the male-specific muscles associated with the proctoderm (obliques and gubernaculum muscles) which in turn bring about contractions in protractor muscles, causing spicule movements (Liu et al., 2011). Dopaminergic signaling is required to inhibit prodding of spicules off the vulva (Correa et al., 2012). The spicule prodding at the precise vulva slit results in partial insertion of spicules. This activates the cholinergic spicule-associated neurons – SPC(L/R), which cause tonic contractions of protractor muscles and complete spicule insertion (Garcia et al., 2001, Garcia and Sternberg, 2003, Liu et al., 2011). The SPD and SPV neurons might be

involved in inhibiting premature ejaculation; however their exact function is still unclear (Liu and Sternberg, 1995, Schindelman et al., 2006). Sperm release is induced by the combined activity of the spicule neurons (SPCs, SPVs, SPDs), p.c.s. neurons and dopaminergic socket cells (LeBoeuf et al., 2014).

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C. elegans locomotion

My dissertation focus is on locomotory changes during mating. In this section, I will describe what is known about the neural control of *C. elegans* locomotion in absence of mate. Our knowledge of the underlying circuitry is based on studies performed in the hermaphrodites. As homologous cells and connectivity exist in the male it is assumed that this system mediates movement in the male in the context of sex-shared locomotory behaviors.

C. elegans *movement patterns*

Many *C. elegans* behaviors involve locomotion. (Chalfie and Sulston, 1981, Hedgecock and Russell, 1975, Way and Chalfie, 1989, Mori and Ohshima, 1995, Fujiwara et al., 2002, Ryu and Samuel, 2002, O'Hagan et al., 2005, Bargmann, 2006, Shtonda and Avery, 2006, Edwards et al., 2008, Ward et al., 2008, Donnelly et al., 2013). To move, the worm lies on its sides and propagates a dorso-ventral wave along its body length. The sinusoidal waveform is generated by alternate contraction and relaxation of body wall muscles that are controlled by distinct set of neurons (Brenner, 1974, White et al., 1986). In absence of specific sensory cues, the worm exhibits so

called “spontaneous locomotion,” which is dominated by forward movement with occasional sharp or shallow turns to change the direction (Gray et al., 2005, Kim et al., 2011). A sharp turn disrupts the sinusoidal waveform as the worm curls it’s head back to touch the tail, forming an Ω shape (omega turns/pirouettes) (Pierce-Shimomura et al., 1999). In a shallow turn, the worm modulates the amplitude of the bends to change direction while maintaining the waveform (Kim et al., 2011). Alternatively, the worm simply reverses (spontaneous reversals), and then resume forward movement in a different direction (Croll, 1975). These various motifs give the worm a fine degree of control over movement in response to external stimuli.

Muscles and motor neurons

The sinusoidal wave is generated by sequential contractions and relaxations of 95 body wall muscles (Sulston and Horvitz, 1977). Sixteen of the 95 body wall muscles are arranged in the head region. These head muscles are innervated by motor neurons located in the head ganglion (associated with the nerve ring) and have complex activation patterns that allow dorso-ventral and lateral head movements. In contrast to the head, the muscles of the body wall have a comparatively simple organization and are oriented longitudinally and are grouped in four quadrants, two on dorsal side and two on ventral side of the body. The location and orientation of the body wall muscles restricts the worm body movement to dorso-ventral plane. The muscles are innervated by the 75 motor neurons located along the ventral nerve cord (VNC) and are called as VNC motor neurons (Sulston and Horvitz, 1977).

The VNC motor neurons can be divided into 8 distinct classes. Four classes (VA, VB, VC and VD) innervate the ventral body wall muscles and remaining four classes (DA, DB, DD and AS) innervate the dorsal body wall muscles (White et al., 1976, White et al., 1986). The A-motor neurons (VA, DA) and B-motor neurons (VB, DB) are cholinergic and excitatory; inducing muscle contraction (Duerr et al., 2008). The D-motor neurons (VD, DD) are GABAergic and inhibitory; inducing muscle relaxation (McIntire et al., 1993).

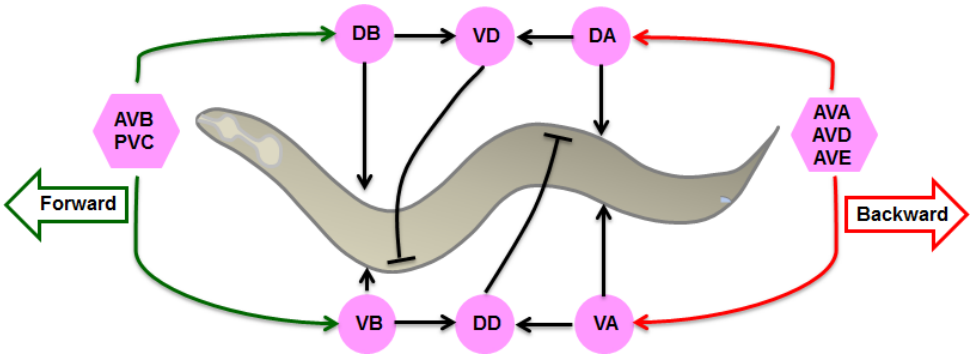

Figure 1.3 The sex-shared *C. elegans* locomotory system.
The sinusoidal wave propagation during worm locomotion is controlled by the VNC motor neurons. A-motor neurons (VAs, DAs) are dedicated to backward locomotion. They are activated by backward command interneurons AVA, AVE and AVD. B-motor neurons (VBs, DBs) are dedicated to forward movement and are activated by forward command interneurons AVB and PVC. A- and B-motor neurons are cholinergic and excitatory, contracting muscles. D-motor neurons (VDs, DDs) are GABAergic and inhibitory, relaxing muscles. The wave is propagated by alternate contraction of the adjacent motor neurons (either A- or B-motor neurons) and cross-inhibition of the D-motor neurons.
(adapted from Chalfie and White, 1988)

The activation of class A-motor neurons produces backward movement and activation of B-motor neurons produce forward movement (Chalfie et al., 1985, Haspel et al., 2010, Kawano et al., 2011). D-motor neurons have an inhibitory action and inhibit the contralateral muscle (White et al., 1986, Donnelly et al., 2013). The locomotory wave is generated by an out of phase contraction-relaxation. For example, when a ventral A- or B-motor neuron induces contraction on the ventral side, the opposite dorsal muscle is relaxed. This cross-inhibition pattern is repeated sequentially along the length of the worm, propagating the sinusoidal wave and producing movement (Fig. 1.3; (White et al., 1986). What controls the direction of wave propagation and thus forward or backward movement is still largely unknown.

Command interneurons

Motor neuron activity is controlled by command interneurons. The command interneurons act as the main center of integration, receiving inputs from various sensory pathways (Tsalik and Hobert, 2003). The backward command interneurons (AVA, AVD & AVE) promote backward movement via regulation of A-motor neurons (VNC motor neurons). The forward command interneurons (AVB & PVC) innervate B-motor neurons and promote forward movement. The respective functions of command interneurons were revealed by systematic laser ablation studies. Elimination of interneurons AVA, AVD and AVE impaired backing and elimination of interneurons AVB and PVC impaired forward movement (Chalfie et al., 1985).

Current model for neural control of locomotion

Recent studies have used a combination of neuronal connectivity, optogenetics and genetics to define the neural mechanisms underlying locomotion at a single cell resolution. In absence of a specific stimulus, the gap-junctional activity between forward command interneurons and B-motor neurons keep the later in an active state , so the worm moves with a forward bias (Fig. 1.4A; (Kawano et al., 2011). Simultaneously, backward movement is inhibited by the RIM interneuron of the disinhibitory pathway (Piggott et al., 2011). Occasionally RIM activity decreases and AVA inhibition is removed, inducing backing and thus spontaneous reversals (Fig. 1.4B). The decrease in RIM activity is due to inhibition by AIB(L/R), a bilateral interneuron pair of head. The reason for this stochastic inhibition by AIB in absence of stimuli, is unknown (Piggott et al., 2011).

When the worm encounters a noxious stimulus, he backs away (Fig. 1.4C). Sensory pathways that detect such cues converge on AIB and backward command interneurons. The stimulation of backward command interneurons and AIB-mediated RIM inhibition shifts the directional bias to backwards (Piggott et al., 2011). The chemical activity between the backward command interneurons AVA and the A-motor neurons also increases the AVA-A gap-junctional activity. The AVA-A gap junctional coupling reduces AVA activity, promoting forward movement (Kawano et al., 2011).

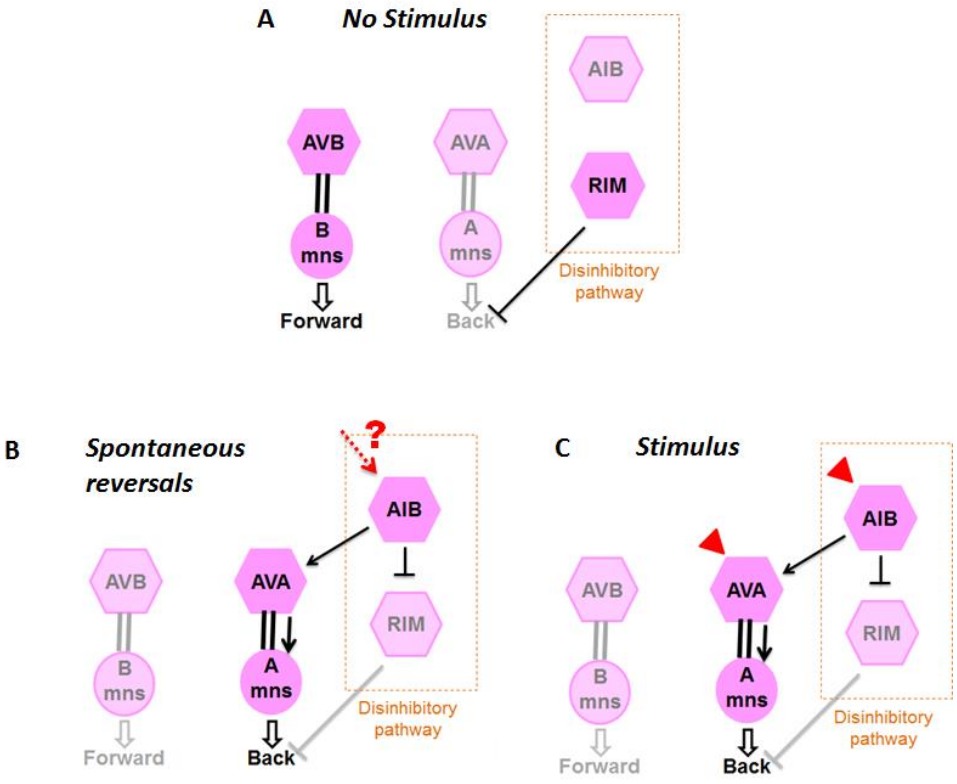

Figure 1.4 Directional control of locomotory motor circuit.
A, B, C. The directional bias of the movement is due to the imbalance in the activities of A-motor neurons (A mns) and B-motor neurons (B mns) created by the gap-junctional properties between the command interneurons (AVB and AVA) and the respective motor neurons with inputs from upstream pathways.
A. In absence of a stimulus, the activity bias is towards B-motor neurons making the worm to move forward which is aided by the inhibition of backing (RIM inhibition) via the disinhibitory pathway.
B. During spontaneous reversals, the AIB activity increases periodically (causes not known) to remove RIM inhibition off backing, leading to reversals.
C. On activation by specific stimulus, the AVA-to-A mns activity increases, shifting the bias towards backward. The same stimulus activates AIB, another component of the disinhibitory pathway to remove the RIM inhibition (disinhibition) from backing.
Key: Sex-shared cells (pink), interneurons (hexagon), motor neurons (circles). Stimulus (arrowheads), unknown stimulus (dotted arrow), stimulatory chemical transmission (arrows), inhibitory transmission (line with a cross bar), gap junctions (two solid bars). Color intensity of lines and shape indicates relative strength of cell activity.
(adapted from Kawano et al., 2011, Piggott et al., 2011)

Dissertation objectives

C. elegans male mating behavior provides a simple paradigm for exploring many relevant processes such as sensory prioritization, execution of behavioral sequences to generate sex-specific behaviors. In spite of knowing the wiring diagram for most of the *C. elegans* male neurons, the exact neuronal pathways involved in modulating the locomotory direction during mating are unknown. Out of 89 male-specific neurons, 85 are located in the posterior region of the male body (Sulston and Horvitz, 1977, Sulston et al., 1980). As described above, male mating behavior is directed by male-specific sensory neurons in the male tail. However, whether these sensory cells use male-specific circuits to produce locomotion in mating or simply modulate activity of the sex-shared locomotory system is unknown. This study set out to define and interrogate the neural pathways that control male movement during his search for the hermaphrodite vulva. Specific questions I wish to address include:

- How does ray contact with a mate induce and sustain the backwards locomotion that characterizes the vulva search?
- What processing strategies enable the male to respond rapidly and accurately to the unpredictable movement of his mate?
- Is vulva search locomotion controlled by male-specific or sex–shared circuitry?
- When the male achieves his goal of locating the vulva, how does the hook terminate locomotion and do so without disrupting tail apposition?

To address these questions I have used a combination of approaches (laser ablation, optogenetics and genetics) and the male connectome as my guide to delineate

the underlying neural ways and interrogate their properties in freely behaving worms. Chapter II describes in detail the experimental procedures used throughout the dissertation. In Chapter III, I have identified the neural pathway controlling the atypical backward movement seen during vulva search. In Chapter IV, I have investigated how hook neurons regulate this pathway to induce pausing at the vulva.

My work reveals that ray neurons use distributed pathways to regulate the sex-shared locomotory systems and distinct molecular signaling mechanisms to impart speed, accuracy and robustness to the male locomotion during mating.

CHAPTER II

EXPERIMENTAL PROCEDURES

Strains

All *C. elegans* strains (Table 2.1) were maintained at 20 ºC under standard conditions as described in Brenner 1974 (Brenner, 1974). *pha-1(e2123ts)* strain was maintained at 15 ºC. All strains have *him-5(e1490)* mutation in the background to generate a high incidence of males (Hodgkin et al., 1979).

Table 2.1: List of strains used

Strains used	References/Source
unc-64(e246)III	Brenner, 1974
unc-29(e193)I	Lewis et al., 1980a, Lewis et al., 1980b
unc-4(e120)II	Miller et al., 1992a
acr-15(ok1214)V	Feng et al., 2006
acr-16(ok789)V	Francis et al., 2005, Touroutine et al., 2005
acr-18(1285)V	Liu et al., 2011
him-5(e1490)V	Hodgkin et al., 1979
lite-1(ce3140)X	Edwards et al., 2008
pha-1(e2123ts)III	Granato et al., 1994
eat-4(ky5)III	Lee et al., 1999
nmr-1(ak4)II	Brockie et al., 2001
glr-1(ky176)III	Maricq et al., 1995
nlp-14(tm1880)X	National BioResource Project

Transgenic arrays

fkEx transgenic lines (Table 2.2) were generated in a *pha-1(ts)* background by Robyn Lints using standard microinjection technique (Mello and Fire, 1995). Transformants survived at 20ºC.

Table 2.2: List of transgenic strains used

Transgenic strain	Genotype	References
akIs3	*Is[Pnmr-1::GFP+lin-15(+)]V*	Brockie et al., 2001
rgIs1	*Ex[Pacr-8(muscle)::unc-29(+)::SL2::GFP]*	Liu et al., 2011
rgEx387	*Ex[Punc-29::unc-29::YFP+pha-1(+)]*	Liu et al., 2011
rgEx196	*Ex[Pacr-18::ChR2::YFP+ pha-1(+)]*	Liu et al., 2011
rgEx575	*Ex[Peat-4::G-CaMP3::SL2::mDsRed]*	LeBoeuf et al., 2014
rgEx431	*Ex[Phsp-16:egl-2(n693gf)cDNA; Punc-103E:mDsRed; pha-1(+)]*	Liu et al., 2011
nIs128	*Is[Ppkd-2::GFP]II*	Yu et al., 2003
fkEx32, fkEx77	*Ex[Pnlp-14(PVY+PVX)::ChR2-YFP+Punc-122::GFP]*	Sherlekar et al., 2013
fkIs6	*Is[Pnlp-14(PVY+PVX)::ChR2-YFP+Punc-122::GFP]*	This study
fkEx63	*Ex[Pnlp-14(PVY+PVX)::mCherry+Pttx-3::mCherry]*	Sherlekar et al., 2013
fkEx66, fkEx67	*Ex[Pnlp-14(PVY+PVX)::NpHR-EYFP+pha-1(+)]*	Sherlekar et al., 2013
fkEx76:	*Ex[Pnmr-1(AVA)::mCherry+Punc-122::GFP]*	Sherlekar et al., 2013

Table 2.2 continued: List of transgenic strains used

Transgenic strain	Genotype	References
fkEx71	*Ex[Pflp-18(AVA)::mCherry+Pttx-3::GFP]*	Sherlekar et al., 2013
fkEx72	*Ex[Pflp-18(AVA)::mCherry+Pnlp-14(PVY-PVX)::ChR2-YFP]*	Sherlekar et al., 2013
fkEx92, fkEx93	*Ex[Pnmr-1(AVA)::acr-18(+)+Pflp-18(AVA)::mCherry]*	Sherlekar et al., 2013
fkEx94	*Ex[Pacr-16::mCherry+Pttx3::mCherry]*	Sherlekar et al., 2013
fkEx95, fkEx96	*Ex[Pnmr-1(AVA)::unc-29(+)::SL2::GFP+Pttx-3::mCherry]*	Sherlekar et al., 2013
fkEx97, fkEx98	*Ex[Ppkd-2::GCaMP V6 medium::SL2::dsRED + pha-1(+)]*	This study
fkEx99	*Ex[Pnlp-14::GCaMP V6 medium::SL2::dsRED + pha-1(+)]*	This study
fkEx106	*Ex[Pflp-7::GCaMP V6 medium-dsRED + pha-1(+)]*	This study

DNA constructs

The Gateway cloning system (Invitrogen) was used to construct all the plasmids. The promoter specific regions were PCR amplified from genomic or plasmid DNA using promoter-specific primers containing attB sites. Below (Table 2.3) are the gene-specific forward (FWD) and reverse (REV) primer sequences used in conjunction with attB1 and attB2 sequences (not shown).

Table 2.3: List of primers used

Gene promoters	Primers	References
nlp-14	FWD: GTTTACCCAGCTTTTTTCATTGTAGAAAACATCAC REV: TGTGCGTGTGTTACCCGGAAAG	Nathoo et al., 2001
flp-18	FWD: GCAAATCTGTCACATACTGCTCGAATCG REV: ACCGTTGCATGTCTAACCCTGAAATTATTA	Kim and Lee, 2011
acr-16	FWD: GATCCGAGAACATGACGATGACAATGATG REV: TACGGACATGAGAATCAGGGAAAGAAAAGC	Feng et al., 2006
nmr-1	FWD: GACACTTTCATCTGTTCAGAATTGAGATGC REV: AACTAAAGTTTGTCGTGTTCCAAACAGAAG	Brockie et al., 2001
pkd-2	FWD: GCTGCAACCAGCAGTATTGTAAATTCGG REV: TGAAGACGGCTCGCTGAAACAGTAG	Barr et al., 2001
flp-7	FWD: ACTCTCCGCTGATTATTCCTCCCCA REV: GAAATGCTTGGATCC CGCTTCCTTC	Kim and Lee, 2011

The attB site bearing PCR fragments were recombined with pDONR221 using Gateway BP clonase to generate the respective entry vectors. The promoter sequences were then recombined with the required destination vectors using Gateway LR clonase II. The destination vectors used in this study are *ccdB C.1::ChR2-YFP* (pLR167) (Koo et al., 2011), *ccdB C.1::NpHR-EYFP* (pZL18) (Sherlekar et al., 2013), *ccdB C.1::mCherry*

(pZL19) (Sherlekar et al., 2013), *ccdB C.1 unc-29(+)::SL2::GFP* (pYL16) (Liu et al., 2011), *ccdB C.1::GCaMP6::SL2::mDsRed* (pLR305) (LeBoeuf et al., 2014).

Laser-mediated cell ablations

PVY, PVX, HOA, HOB, AVG ablations at L4: L4 males were mounted on 10% agar pads with 0.25µL of polystyrene beads (Fang-Yen et al., 2009) and minimal amount of M9. PVY and/or PVX ablations were performed on males carrying *fkEx32* or *fkEx77 [Pnlp-14(PVY+PVX)::ChR2-YFP+Punc-122::GFP]* transgenic arrays. HOA ablations were performed on male carrying *rgEx575[Peat-4::G-CaMP3::SL2::mDsRed]* transgenic array. HOB ablations were performed on male carrying *nIs128 [Ppkd-2::GFP]* transgenic array. AVG ablations were performed on male carrying *fkEx106 [Pflp-7::GCaMP V6 medium-dsRED + pha-1(+)]* transgenic array. Fluorescent expression (PVY/PVX-YFP; HOA-GCaMP; HOB-GFP; AVG-GCaMP) was used to identify the cells for ablations. Control males were mounted on the 10% agar pads with polystyrene beads and M9 but not subjected to laser treatment. Post-ablation males were put on a separate plate from hermaphrodites, allowed to recover and grow into adults for approximately 24 hours. Mating assays or ChR2 assays were performed as described below. To confirm cell death, absence of fluorescence was checked under Zeiss D1 compound microscope after mating and/or ChR2 assays. Only data from animals with confirmed cell death were used for subsequent analysis.

EF neuron ablations at L1: L1 animals were mounted on 5% agar pads with 2mM sodium azide (NaN_3) used as an anesthetic (Bargmann and Avery, 1995). The ablations

of precursor cells F and U were carried out in males carrying *fkIs6 [Pnlp-14(PVY+PVX)::ChR2-YFP+Punc-122::GFP]* array or in *him-5* mutant males. F and U were identified using DIC (Sulston et al., 1983) and laser ablated at L1 stage. The loss of F and U cells in males was confirmed by crumpled state of spicules.

AVA ablations at L1 and L4: AVA interneurons were ablated at L1 stage. L1 animals were mounted put under anesthetic as described in EF ablations. AVA ablations were carried on males carrying *fkEx71 [Pflp-18(AVA)::mCherry+Pttx-3::GFP]* or *fkEx72 [Pflp-18(AVA)::mCherry+Pnlp-14(PVY-PVX)::ChR2-YFP]* arrays. FLP-18::mCHERRY expression was used to identify the AVA cells. Control males were also mounted on agar pads with anaesthetic but not exposed to laser treatment. AVA neurons are difficult to ablate and even after subjecting to laser treatment, sometimes there is residual fluorescence suggesting the cell is not completely destroyed.To ensure that the cells were completely eliminated, L1 males were allowed to grow to for day and remounted on agar pads with anaesthetic to check FLP-18::mCHERRY expression in AVA. If there was mCHERRY expression then the cells were re-ablated. The ablated and control animals were allowed to grow to L4 stage and then males were separated from hermaphrodites. These virgin males were allowed to grow into adults and subjected to mating and/or ChR2 assays. After assays, cell ablations were confirmed by checking for absence of fluorescene under compound microscope.

Mating behavior assays

Mating assays were performed as per procedures described in (Liu et al., 2007).

Preparation of animals for mating assays: The experimental males (10 per plate) were picked at L4 stage, a day before the assays and allowed to mature into adults, separate from hermaphrodites so they were virgins and of the same age. Similarly, virgin L4 hermaphrodites (20 per plate) were picked and allowed to mature overnight into adults.

Preparation of mating plate: On the day of assays, 1 mL of OP50 was centrifuged for 5 minutes at 6000 rpm. The supernatant was discarded leaving 50uL for resuspending the pellet. 5uL of the concentrated suspension was spotted on a fresh plate to make a mating lawn of approximately 5mm in diameter and allowed to soak in.

Mating assays: For the ablation and mutant studies, 5 *unc-64; lite-1* virgin 1-day old adult hermaphrodites were placed on the lawn. After 5 minutes (to allow the hermaphrodites to settle), one experimental male was placed on the mating lawn. The mating behavior was recorded using a Zeiss AxioCam HS digital camera and AxioVision software (release 4.7) for 15 minutes or until the male ejaculated, whichever occurred first.

Mating behavior analysis: The videos were analyzed for execution of different motor behaviors during mating. The efficiency of vulva search was measured by quantifying contact respone, scanning speed and loss of tail contact during scanning.

% Contact Response = 100 x [the number of times the male exhibits contact response/the number of times the ventral part of the male tail makes contact with a hermaphrodite]. A successful (complete) contact response involves both tail apposition and initiation of backwards locomotion (Koo et al., 2011).

Scanning speed on the non-vulva side(μm/sec) = average length of 1-day old adult *unc-64; lite-1* hermaphrodites (1044 μm)/ the time required for a male to travel the length (sec). The average speed for a male was calculated from a random selection of 5 non-vulva sides scanned, or all non-vulva sides scanned if the number of sides completed was less than 5.

Loss of tail contact = the number of times a male lost tail contact with the hermaphrodite during the mating trial while scanning.

The efficiency of scanning was measured by the frequency of pausing = number of times the male pauses while scanning the non-vulva side of the hermaphrodite during a mating trial.

ChR2 & NpHR assays

ChR2 assays: All strains, except for the –ATR controls were grown on plates seeded with *E.coli* OP50 containing 50μM ATR. Since ATR is light sensitive, all plates were kept in dark by wrapping them in a foil. A day before the assays, five L4 males or hermaphrodites were placed on separate +ATR (spread with *E.coli* OP50 and 50μM ATR) or –ATR (spread with *E.coli* OP50 only) plates to mature. The worms were allowed to mature overnight into adults. Assays for individual worms were recorded using Zeiss AxioCam HS digital camera and AxioVision software. A Zeiss M2 Imager stereomicroscope with epi-fluorescence was used to perform the assays. For ChR2 assays, each worm was exposed to three, 500 msec flashes of blue light (470/40 nm in wavelength), when they were moving forward. The flashes were evenly spaced allowing

enough time in between for recovery from the previous flash. Mostly, response to the first flash was quantified (as described in the following section). For PVY/PVX activation, *fkEx32, fkEx77 or fkIs6 [Pnlp-14(PVY+PVX)::ChR2-YFP+Punc-122::GFP]* strains were used. After the assays, PVY and PVX expression for ChR2-YFP was verified in each worm at 600X magnification using Zeiss D1 compound microscope equipped with epi-fluorescence. Only data from animals with expression in both the cells were used for subsequent analysis.

NpHR assays: Worms were maintained under similar conditions as described for ChR2 assays (NpHR also requires ATR for functional activity). For PVY/PVX inhibition, *fkEx66 or fkEx67 [Pnlp-14(PVY+PVX)::NpHR-EYFP+pha-1(+)]* strains were used. The day before the assays, ten L4 males were placed on separate plates spread with *E.coli* OP50 (with ATR and without ATR). For NpHR assays with solitary males, each worm was exposed to five, 500msec flashes of yellow light (540/25 nm in wavelength) with evenly spaced intervals while the male was moving in forward direction. Pausing on light exposure was calculated as follows:

% pauses with light flash = 100 X [number of pauses on light flash/5(total number of flashes)]

For NpHR assays in context of mating, a one-day old adult, virgin male was placed on a mating lawn with 5 one-day old adult, virgin *unc-64; lite-1* hermaphrodites. When the male started backing on a hermaphrodite, he was exposed to five, evenly spaced flashes of yellow light. The percentage for number of pauses induced out of five was measured.

Rescue experiments

The experiments for rescuing *acr-18* and *unc-29* function in AVA interneurons were performed by Robyn Lints.

For *acr-18* rescue: *acr-18; fkEx92* or *fkEx93 [Pnmr-1(AVA)::acr-18(+)+Pflp-18(AVA)::mCherry]* hermaphrodites were crossed with *acr-18; fkEx32* or *fkEx77 [Pnlp-14(PVY+PVX)::ChR2-YFP+Punc-122::GFP]* males. L4 stage F1 cross progeny males identified as being UNC-122::GFP (ChR2 in PVY/PVX) and FLP-18::mCHERRY (acr-18(+) in AVA) positive, were transferred to plates (5/plate) spread with E.coli OP50 and 50µM ATR. The males were allowed to mature overnight into adults. Wild type and *acr-18* m*ales* carrying transgene *fkEx32* or *fkEx77* were used as positive and negative controls respectively. ChR2 assays were performed on each male as described above. After assays, each individual male was checked for CHR2-YFP expression in PVY and PVX; and mCHERRY expression in AVA interneurons (Sherlekar et al., 2013).

For *unc-29* rescue: *unc-29; acr-16; rgIs1[Pacr-8(muscle)::unc-29(+)::SL2::GFP]*; *fkEx95* or *fkEx96 [Pnmr-1(AVA)::unc-29(+)::SL2::GFP+Pttx-3::mCherry]* hermaphrodites were crossed with *unc-29; acr-16; fkEx32 or fkEx77 Ex[Pnlp-14(PVY+PVX)::ChR2-YFP+Punc-122::GFP]* males. The F1 progeny L4 males with UNC-122::GFP (ChR2 in PVY/PVX) and TTX-3::mCHERRY (*unc-29 (+)* in AVA) expression were selected for ChR2 assays. The assays were performed as described in *acr-18* rescue experiments (Sherlekar et al., 2013).

ChR2 assay quantification

In ChR2 assays, the response to blue light flash was digitally recorded as described in the above sections. The "start of flash" was easily tracked by the fluorescence of coelomocytes (Fig. 2.1A). After the flash, the first frame where the male started backing was extracted from the movie using Axiovision software tool and labeled "start of response" (Fig. 2.1B). The frame wherein the male stopped backing was also extracted and labelled "end of response" (Fig. 2.1C). For each frame, the distance of the male head (or tail, keeping the head/tail parameter consistent for one given worm) was measured

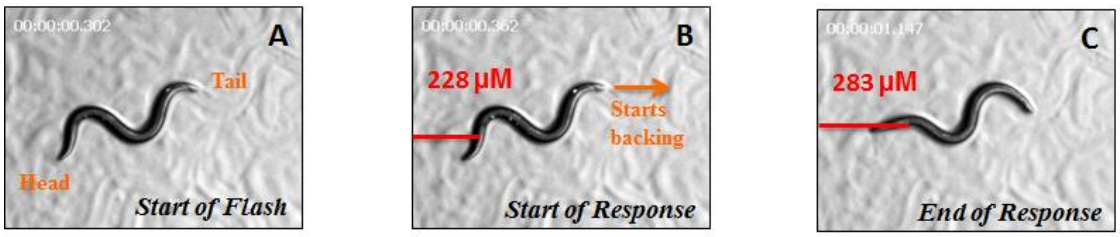

Figure 2.1 Quantification of locomotory response.
A, B, C. Micrographs of adult male showing different stages in ChR2-mediated activation of PVY/PVX.
A. "Start of flash" frame. Head is on the left and tail is on the right side. The top left corner shows the time stamp. The fluorescent dots on the worm body is the coelomocyte fluorescence in response to blue light, used as a marker to identify the start of flash.
B. "Start of response" frame. The male starts moving backwards in response to the light flash. To locate the position of the male at "start of response", a line (in red) is drawn using Axiovision software from the head to the left edge (nearest edge to the male head). The distance is measured in µm.
C. "End of response" frame. The distance at "end of response" is measured similarly as described for "start of response" frame. Distance moved in µm = length in "start of response" frame – length in "end of response" frame.

from the closest edge of the frame. The "distance moved" (in µm) = length in "start of response" frame – length in "end of response" frame (In Fig. 2.1, distance moved = 228 – 283 = -55 µm).

The average time to respond (time to respond in secs = time at "start of response" – time at "end of response") and the average time of response (response time in secs = time at "end of response" – time at "start of response") was calculated from the animals who responded by backing on exposure to light flash. These values were then plugged in to the "start of flash" time for the non-responders to extract "start of response" frame and "end of response" frame. The distance moved for these animals was in forward direction and gave a positive value. Based on the "distance moved" values scored, the experimental animals were categorized into three different groups; backing = distance travelled < 0 µm, pausing = distance travelled 0 to +2.5 µm, forward = distance travelled > +2.5 µm.

GCaMP assays

The strains used to study Ca^{2+} transients carried the following transgenes:

PVY activity - *fkEx99 [Pnlp-14::GCaMP V6 medium::SL2::dsRED + pha-1(+)]*

HOB activity - *fkEx97 or fkEx98 [Ppkd-2::GCaMP V6 medium::SL2::dsRED + pha-1(+)]*

AVG activity - *fkEx106 [Pflp-7::GCaMP V6 medium-dsRED + pha-1(+)]*

A day prior to the assays, the experimental males were picked at L4 stage and allowed to mature overnight. The hermaphrodites used in these assays were of the

genotype *rgEx431[Phsp-16:egl-2(n693gf)cDNA; Punc-103E:mDsRed; pha-1(+)]*. On the previous day, hermaphrodites were heat shocked for 2-3 hrs at 33ºC. The overexpression of EGL-2 on heat treatment of *hsp-16* promoter, completely paralyze the hermaphrodites. UNC-103E::dsRED expression marks the vulva location. The mating lawns were prepared as described in the mating behavior assays section. On the day of assays, eight 2-day old heat shocked hermaphrodites were placed on the mating lawn, allowing them enough time to settle (10-15 minutes). A 2cm square containing the mating lawn was cut and placed on a microscope slide. An individual one-day old male was then placed on the mating lawn. The mating behavior was recorded after the male came in the vicinity of the hermaphrodites on a fluorescene equipped Olympus BX52 microscope. The GCaMP and DsRed signals were visualized and recorded simultaneously using a Dual View Simultaneous Imaging Systems with an Ol-11-EM filter and Hamamatsu ImagEM Electron multipier (EM) CCD camera. The mating behavior movies were analyzed for the activity of PVY, HOB and AVG neurons in response to vulva location.

The mating movies were cut in to smaller recordings which included the approach of male tail to the vulva and the response on vulva location. The Hamamastu SimplePCI software was used to locate region of interest (ROI); centered on the neurons to be studied (PVY or HOB or AVG) in both GCaMP and dsRed channels. A neutral ROI was selected from the bacterial lawn to record background fluorescence. The recording was analyzed on frame-by-frame basis, to score the mean gray levels for all the three ROIs. In case of any movement, the ROIs were adjusted to center on the neurons of interest.

The neutral ROI is kept at the same position in all the frames. The data for all the frames for a given recording is then transferred to a Microsoft Excel sheet to calculate % ΔF/F0 (Correa et al., 2012). F0 is the fluorescent intensity of dsRed which is used as a baseline and corrects any discrepancies caused by focal plane changes or any other imaging artifacts. ΔF is the difference between the fluorescent intensity of GCaMP (F) and the baseline (F0). The neuronal activity can be seen by plotting % ΔF/F0 (on Y-axis) against time (on X-axis).

Statistical analysis

All the groups were compared to the control or wild type groups using Wilcoxon-Mann-Whitney ranksum test.

CHAPTER III

THE NEURAL AND MOLECULAR MECHANISMS REGULATING BACKWARD MOVEMENT DURING THE VULVA SEARCH

Sex-shared backward command interneurons control locomotion during mating

C. elegans male mating behavior is initiated when the male tail contacts a potential mate. The contact prompts the male to cease forward locomotion, appose his tail against the hermaphrodite surface and start backing along the cuticle in search of the vulva. The sensory rays in the male tail induce and guide the search by promoting two sub-behaviors: backward movement and tail apposition (Koo et al., 2011). The male tail connectivity shows that sensory ray neurons connect to male-specific interneurons in the pre-anal ganglion which in turn form connections to backward and forward command interneurons. The connectivity weightage favors the backward command interneurons, especially AVA(L/R) (Fig. 3.1; Sherlekar et al., 2013). To test whether male backing during mating is controlled by AVA interneurons, I laser ablated these cells in males and assessed its impact on mating behavior. AVA interneurons are present from the embryo stage; however presynaptic male-specific cell types in the tail do not form or connect until larval stage L4. Hence, the cell-specific laser ablations were done in L4 males. To aid identification of AVA and assess the effectiveness of cell killing, I used transgenic strain in which AVA was marked with a reporter (*Pflp-18(AVA)::mCherry*). Mock-ablated males were exposed to anaesthetic and mounted on the slide but were not subjected to laser (refer Experimental Procedures). Mock and laser-ablated males were

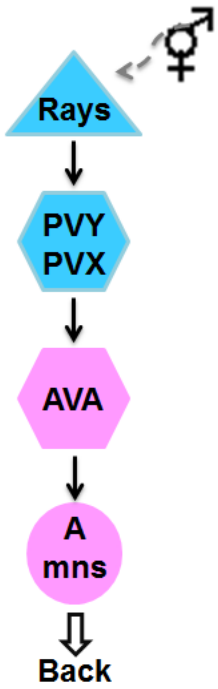

Figure 3.1 Putative backing circuit.
Wiring diagram of the circuit model being tested (based on male tail connectivity; (Jarrell et al., 2012). The rays have heavy connections to male-specific interneurons PVY/PVX, which have major connections to backward command interneurons, AVA(L/R). In sex-shared locomotory circuit, AVA interneurons activate VNC A-motor neurons to promote backing.
Key: Sex-shared cells (pink), male-specific (blue), sensory neurons (triangles), interneurons (hexagon), motor neurons (circles). Stimulus (dotted arrow), stimulatory chemical transmission (arrows).

allowed to recover and mature overnight into adults in absence of hermaphrodites. For some unknown reason, AVA neurons were difficult to ablate as evidenced by persistent fluorescence the next day. If still present, the cells were laser ablated again. Mock-ablated and laser ablated animals were allowed recovery for more than 5 hours. Mating assays were performed with *unc-64* hermaphrodites, which are paralyzed due to a

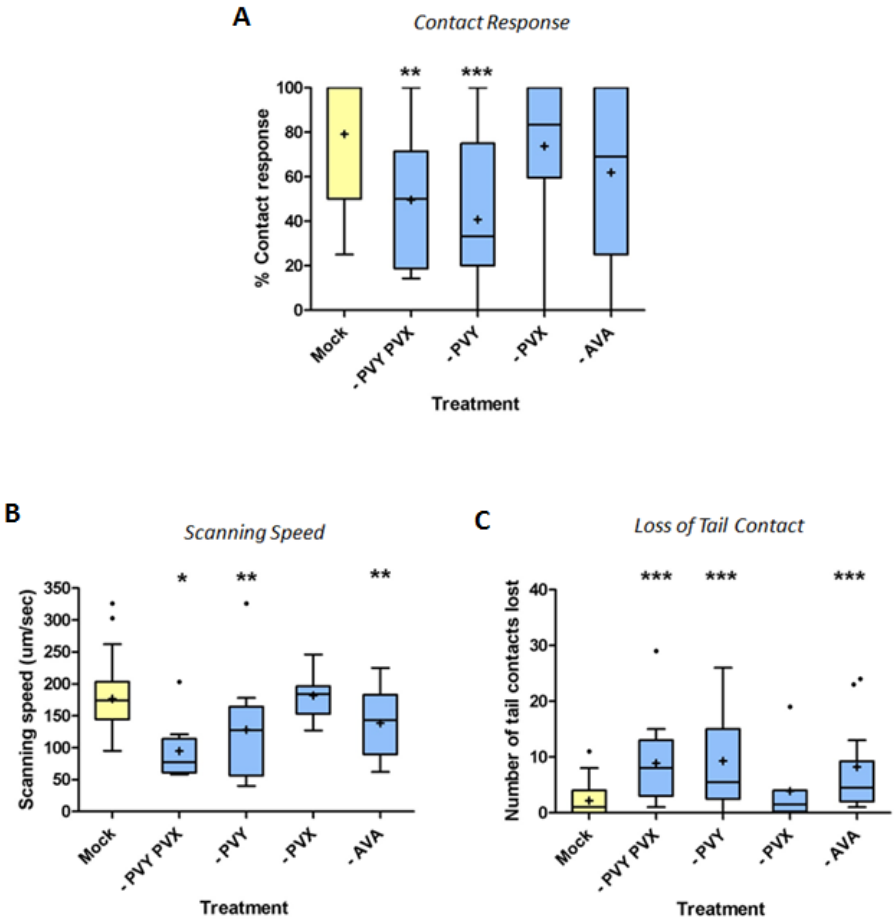

Figure 3.2 Male-specific and sex-shared interneurons control direction of male locomotion during mating.

A, B, C. The impact of cell-specific ablations on the initiation and maintenance of vulva search behavior.

A. Contact response (in %) measures the efficiency of the male to initiate mating response. A successful contact response requires both tail apposition and initiation of backward movement along the hermaphrodite (Koo et al., 2011).

B-C. The efficiency of scanning on the hermaphrodite.

B. Scanning speed (in µm/sec) is the speed with which the male scanned the hermaphrodite length.

C. Loss of tail contact indicates the number of times the male lost contact during mating.

A box-plot representation of the data shown, with median and mean values indicated by a line and a plus "+" sign, respectively. n for each treatment group: Mock=63; -PVY-PVX=15; -PVY=19; -PVX=8; -AVA=22. Statistical comparisons were made using ranksum test for differences in median. Significance, *p <0.05, **p <0.005, ***p <0.001

mutation in the syntaxin gene (Saifee et al., 1998). Both the males and hermaphrodites were virgin, 1-day old adults. For the mating assay, one male was put on a mating lawn with 5 hermaphrodites and the assay was recorded for 15 minutes or until the male ejaculated (Koo et al., 2011). The mating behavior was analyzed to determine the ability of the male to induce and maintain backing. Contact response was measured to see if the male could initiate the backing response. The efficiency of backing was determined by quantifying scanning speed and loss of tail contact during scanning. Scanning speed measurements indicated the strength of backing response and loss of tail contact during scanning measured the ability of the male to keep scanning and to maintain mating postures.

A successful contact response constitutes pressing of the male tail against the hermaphrodite cuticle and initiation of backing. Mock-ablated males show 100% (median) efficiency in contact response (Fig. 3.2A; Sherlekar et al., 2013), *i.e.* virtually all contacts resulted in a complete contact response. On completion of the contact response males initiated a systematic search of the hermaphrodite cuticle in search of the vulva. Typically the search would involve scanning and turning until the vulva was located. Scanning speed (μm/sec) was measured by dividing the average length of the hermaphrodites by time taken by a male to scan from one end to the other. If the male encountered the vulva, typically he stopped there. This pausing prolonged the time taken to scan the vulva side of the hermaphrodite. So, all the scanning speed measurements were taken on the non-vulva side of the hermaphrodite. Mock-ablated males scanned the non-vulva side at the speed of 180μm/sec (Fig. 3.2B; Sherlekar et al., 2013). Another

important feature of the male scanning is that the ventral side of the male tail is continuously in contact with the hermaphrodite throughout the mating behavior. To determine whether the male could maintain contact during scanning and turning, I counted the number of times the male lost contact with the hermaphrodite (ventral side of the tail came off). Mock-ablated males were efficient in maintaining contact with the hermaphrodite, having a median of 1 loss of tail contact during the vulva search (Fig. 3.2C; Sherlekar et al., 2013).

AVA-ablated males showed defects in reversing. When not engaged in mating, similar to hermaphrodites AVA-ablated hermaphrodites (Chalfie et al., 1985), these solitary males showed difficulties in switching from forward to backward direction. Males spent most of the time spent moving in the forward direction or pausing during attempts to reverse. Males also exhibit locomotory defects during mating. The AVA-ablated males showed varied contact response though not significantly different from the controls (Fig. 3.2A; Sherlekar et al., 2013), indicating that they can sense the hermaphrodite and try to initiate the backing behavior. However, the other two parameters which measure the strength and ability of backing were clearly more affected. AVA-ablated males had average scanning speeds of 140µm/sec, significantly slower than the control males (180µm/sec; Fig. 3.2B; Sherlekar et al., 2013). Also, AVA-ablated males had difficulty in maintaining contact with the hermaphrodite; a loss of contact median of 5 *cf.* control male 1 (Fig. 3.2C; Sherlekar et al., 2013). These latter defects can be attributed to the uncoordinated phenotype of the AVA-ablated males. Taken together, these results suggest that the sensory rays use sex-shared backward

command interneurons to induce and maintain backing during the search. However, as the AVA-ablated males can still back stochastically; other neural pathways may also contribute.

Male-specific interneuron PVY promotes backward locomotion during mating

The next question was how do sensory rays activate the backward command interneurons. Sensory rays have sparse direct connections to backward command interneurons; however, they are heavily connected to AVA interneurons via male-specific interneurons PVY and PVX (Fig. 3.1; Jarrell et al., 2012; Sherlekar et al., 2013). To determine if PVY and PVX represent a key connection between the rays and AVA, I laser ablated PVY and/or PVX. PVY differentiates at L3 stage; hence the PVY and PVX ablations were done at L4 stage and ablated males were allowed to mature overnight on a separate plate. Mating assays were performed as described for AVA ablations.

Males ablated for PVY only showed defects in mating behavior. They had difficulty in completing the contact response, with an efficiency median of 30% as compared to control males with 100% efficiency median (Fig. 3.2A; Sherlekar et al., 2013). The few times PVY-ablated males were able to initiate backing they exhibited slow scanning speed (135µm/sec as compared to 180µm/sec of control males; Fig. 3.2B; Sherlekar et al., 2013). In addition, PVY-ablated males could not maintain contact with the hermaphrodite. The median number of times males lost tail contact was 5 as compared to a 1 for control males (Fig. 3.2C; Sherlekar et al., 2013). These mating defects are similar to AVA-ablated males consistent with the hypothesis that rays, PVY

and AVA form a pathway. Males ablated for both PVY and PVX showed similar defects to PVY only ablated males. By contrast, PVX-ablated males were not significantly different from control males, suggesting that PVX is either functionally redundant with PVY or that it has no role in the vulva search locomotion.

Together with the wiring data (Jarrell et al., 2012), these results argue that sensory rays modulate backward command interneurons AVA through male-specific interneuron PVY to promote backing during mating. In contrast to AVA-ablated males, males lacking PVY showed no obvious defects in locomotion when they are not mating. This suggests that PVY mediated AVA activation only occurs in the context of mating. The slow and erratic backing in AVA- and PVY-ablated males strongly suggest that the sensory rays–to–PVY–to–AVA pathway is the major pathway that controls backing during mating. However, as in AVA-ablated males, PVY-ablated males show stochastic backing behavior suggesting that sensory rays might also utilize other pathway to control movement. This alternate pathway will be examined later in this chapter.

Artificial activation of PVY induces backward movement in solitary males

To confirm the role of PVY and further explore if PVX has any role in backing, I artificially activated PVY and/or PVX using the light-gated channel Channelrhodopsin (ChR2) from *Chlamydomonas reinhardtii* (Nagel et al., 2005). ChR2 is a cation channel, activated on exposure to blue light and in presence of its essential co-factor all-*trans*-retinal (ATR). The blue light (approximate wavelength 450nm) brings about conformational change in the retinal molecule by changing it from all-*trans*-retinal to

all-*cis*-retinal ultimately causing the channel to open. The open channel allows the influx of cations which in turn depolarizes the neuron membrane and activates the neuron. Previous experiments in our lab, using *Pnlp-14::GFP* (Nathoo et al., 2001) transgene showed NLP-14::GFP expression in PVY and PVX. To target PVY and PVX, *ChR2* transgene was placed under the control of *nlp-14* promoter (Nathoo et al., 2001). The ChR2 transgene was tagged with YFP (*ChR2::YFP*) allowing visualization of cells expressing the channel. ATR is an essential co-factor, required for ChR2 activity. However, *C. elegans* does not produce ATR endogenously so it was supplemented with the food. All the experimental animals (except for "-ATR" males) were grown in presence of ATR. In absence of ATR, ChR2 is non-functional and ChR2 transgenic strains grown without ATR can be used as an internal control for any non-specific responses to blue light exposure such as escape response, which involves reversals (Edwards et al., 2008).

ChR2 assays were performed on virgin, one-day old adults. The experimental animal was exposed to a 500msec flash of blue light when moving in a forward direction. The response was quantified by measuring the distance travelled in response to the light flash. A negative distance value indicates movement in backward direction and a positive value indicates forward movement (see Experimental Procedures). In presence of ATR, *Pnlp-14::ChR2-YFP* males reverse immediately after the blue light flash. Ninety percent animals from this group backed with a median distance of -5µm ("Males" in Fig. 3.3A; Sherlekar et al., 2013).

The following sets of controls confirm that the reversal response was specific to PVY and PVX activation. *Pnlp-14::ChR2-YFP* males grown in absence of ATR have non-functional ChR2 and did not back in response to blue light. The "-ATR" males moved a median distance of 7µm in forward direction ("-ATR Males" in Fig. 3.3A; Sherlekar et al., 2013). The expression pattern for *nlp-14* reporter gene shows expression

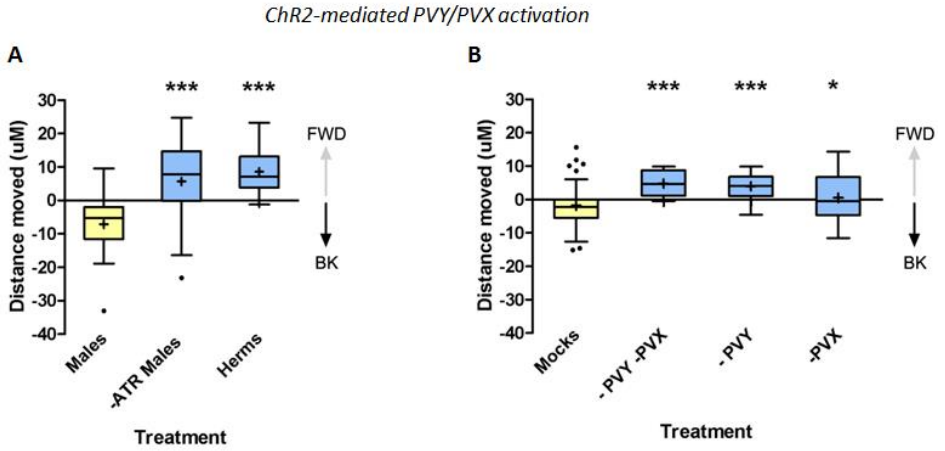

Figure 3.3 PVY activity is sufficient to induce backing.
A, B. Artificial activation PVY/PVX using ChR2, induces backward movement. All the animals carry the transgene *Pnlp-14(PVY,PVX)::ChR2-YFP*. The graphs depict the distance moved (in µm, Y-axis) on cell-specific ChR2 activation (500msec flash with blue light). The "distance moved" (in µm) = coordinate in the "start of response" frame – coordinate in the "end of response" frame, where backward movement (BK) has a negative value and forward (FWD) movement has a positive value. The X-axis indicates worm sex, growth conditions *(A)* and cells ablated *(B)*. Except for herms (hermaphrodites) in *(A)* all animals tested are males. Except for "-ATR Males" treatment, all animals tested were grown on ATR supplemented OP50 *E. coli* (food). In *(B)*, the controls for ablated animals are indicated by "Mock".
A box-plot representation of the data shown, with median and mean values indicated by a line and a plus "+" sign, respectively. Statistical comparisons were made using ranksum test for differences in median. Significance, *p <0.05, ***p <0.001

in sex-shared neurons ASI, ASK, ASE, PHA, PVT, two retrovesicular ganglion neurons and ventral nerve cord motor neurons (Nathoo et al., 2001). *Pnlp-14::ChR2-YFP* males lacking PVY and PVX (due to their laser ablation) did not reverse in response to blue light in presence of ATR ("-PVY PVX" in Fig. 3.3B; Sherlekar et al., 2013), eliminating contributions from other ChR2 expressing neurons in backing. *Pnlp-14::ChR2-YFP* hermaphrodites grown in presence of ATR which lack PVY and PVX but express ChR2 in the sex-shared cells, did not reverse on flashing with blue light ("Herms" in Fig. 3.3A; Sherlekar et al., 2013). Collectively, these results confirm that PVY and potentially PVX activation is sufficient to induce reversal in solitary males.

To further dissect the role of PVY and PVX, I activated only PVY or PVX by laser ablating PVX or PVY, respectively. Consistent with their impact on mating behavior, PVY was primarily responsible for driving backward movement. By, contrast activating PVX alone, did not promote reversal ("-PVY" in Fig. 3.3B; Sherlekar et al., 2013). However, its activation without PVY greatly increased reversal robustness ("-PVX" in Fig. 3.3B; Sherlekar et al., 2013). These results indicate that PVY is the major effector in the PVY/PVX induced reversals and that PVX may serve an auxiliary function. In the context of mating, other ray targets might be functionally compensating loss of PVX.

PVY, PVX inputs modulate sex shared backward locomotory circuit to induce backing

My next step was to confirm whether PVY and PVX induced reversals are dependent on sex-shared locomotory circuit. To test this, I disrupted the activity of

specific sex-shared cell types: AVA interneurons (by means of laser ablations) and the A type motor neurons of the ventral nerve cord (VNC) (by genetic means). Activation of PVY/PVX in AVA-ablated males resulted in little or no reversal (Fig. 3.4; Sherlekar et al., 2013). Thus, PVY/PVX induced reversals depend on AVA activation.

The backward command interneurons induce reversals by activating a distinct set of VNC motor neurons called A-motor neurons which control the body wall muscles to

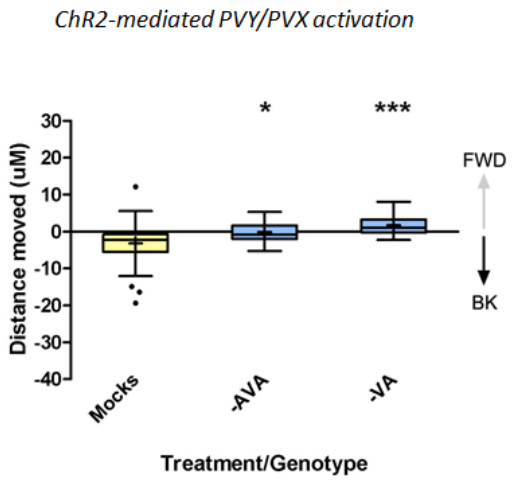

Figure 3.4 PVY/PVX induced backing during mating depends on sex-shared backward locomotory circuit for activity.
Males with impaired sex-shared backward locomotory circuit have defects in PVY/PVX induced backing. All the animals carry the transgene *Pnlp-14(PVY,PVX)::ChR2-YFP* for artificial activation of PVY/PVX. See legend for Fig. 3.3. The X-axis indicates Treatment/Genotype; cells ablated/defective. The controls are indicated by "Mock".
A box-plot representation of the data shown, with median and mean values indicated by a line and a plus "+" sign, respectively. Statistical comparisons were made using ranksum test for differences in median. Significance, *p <0.05, ***p <0.001

induce backing. To confirm PVY/PVX induced reversals are dependent on A-motor neurons, I activated PVY/PVX in *unc-4* mutant males. In *unc-4* mutants, the ventral A-motor neurons are mis-specified and incorrectly wired as B-motor, the motor neurons responsible for forward movement (Miller et al., 1992a). I observed that, only 27% *unc-4* mutant males backed (and less than 1µm) on PVY/PVX activation (Fig. 3.4; Sherlekar et al., 2013). Taken together, these results suggest that PVY/PVX induced reversals are dependent on sex-shared backward command interneurons AVA and backward A-motor neurons.

PVY is required continuously while backing on the hermaphrodite

The above experiments suggest that PVY is sufficient to induce the directional change during mating behavior and PVX playing an auxiliary but non-essential role (Fig. 3.3B; Sherlekar et al., 2013). To further investigate whether PVY activity (and that of PVX, since *nlp-14* reporter gene in expressed in both cells) is also required to maintain the backward movement throughout the vulva search, I inhibited PVY/PVX activity in context of mating, specifically while the male was scanning the hermaphrodite. PVY/PVX activity was inhibited by using NpHR, a chloride pump from *Natrosomonas pharaonis* which hyperpolarizes the cell on activation by yellow light (540nm) (Zhang et al., 2007, Zhao et al., 2008). Similar to ChR2, NpHR activity depends on co-factor ATR, which was supplemented through the worm food. The males grown in absence of ATR had non-functional NpHR and were used as controls to eliminate the possibility of

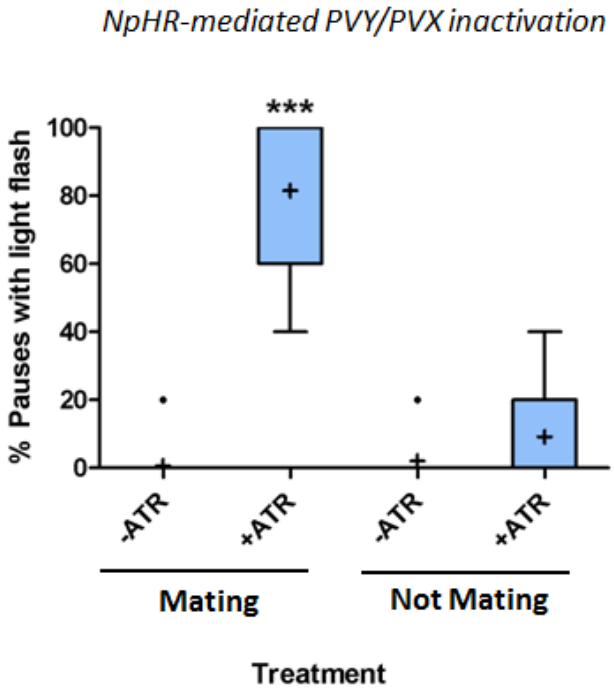

Figure 3.5 PVY, PVX are required continuously for backing in context to mating.
Artificial inactivation of PVY/PVX using NpHR, blocks backing during mating. All the males carry transgene *Pnlp-14(PVY,PVX)::NpHR-EYFP*. The X-axis indicates treatment (food supplemented with ATR "+ATR" or without ATR "-ATR") and mating conditions used ("Mating" or "Not Mating"). In *Mating*, assays performed while male was backing on the hermaphrodite and in *Not Mating*, assays were performed on solitary males. The Y-axis indicates the percentage of flashes (out of 5 evenly spaced flashes) the males stopped backing. n for each group: -ATR mating=34; +ATR mating=33; -ATR Not Mating=10; +ATR Not Mating=11. Statistical comparisons were made using ranksum test for differences in median. Significance, ***p <0.001

yellow light affecting the male behavior. NpHR was targeted to PVY and PVX by placing a *NpHR-EYFP* transgene under the control of *nlp-14* promoter. As per standard mating assays, a one-day old virgin male was placed on a mating lawn with 5 one-day old adult virgin hermaphrodites and allowed to initiate backing. While backing on the

hermaphrodite, the male was exposed to a 500msec flash of yellow light and the response was recorded. Each male was given 5 such flashes at evenly spaced intervals and the total number of light-induced pauses was counted. The males with active NpHR in PVY and PVX paused in 4 out of 5 flashes, giving an average mean frequency of 80% for all the experimental animals ("+ATR Mating" in Fig. 3.5; Sherlekar et al., 2013). However, the "- ATR" males, with non-functional NpHR, did not respond to the yellow light flash and paused with an average mean frequency of 1% ("-ATR Mating" in Fig. 3.5; Sherlekar et al., 2013). The presence of NpHR in PVY and PVX was confirmed by checking the YFP tag expression after the assays. The next question was whether the NpHR-mediated PVY/PVX inactivation is specific to the mating behavior. To test this, solitary *Pnlp-14::NpHR-EYFP* males grown in presence of ATR where exposed to 500msec yellow light flashes while they were moving in forward direction. These males seldom paused, having an average mean frequency of 10% ("+ATR Not Mating" in Fig. 3.5; Sherlekar et al., 2013). As NpHR-mediated inhibition has no impact on behavior, this suggests that PVY and PVX neurons are not active in non-mating male, only in the context of mate-contact.

The above data was further supported by our examination of PVY activity during mating using the Ca^{2+} sensor GCaMP. GCaMP is a heterologous Genetically Encoded Calcium Indicator (GECI) consisting of EGFP (Enhanced Green Fluorescent Protein), calmodulin or CaM (Ca^{2+} binding peptide) and M13 (containing target sequence for CaM binding). Ca^{2+} binds to CaM, which in turn interacts with M13 resulting in conformational changes in EGFP-CaM-M13 complex (Nakai et al., 2001, Tian et al.,

2009). The conformational change in EGFP increases the fluorescence intensity of the protein which can be recorded and quantified. An activated cell is in a depolarized state, which is caused by increase in influx of cations. GCaMP binds to Ca^{2+} and its level of fluorescent intensity is proportional to Ca^{2+} concentration in the cell. GCaMP expression was targeted to PVY/PVX using *nlp-14* promoter. The *GCaMP* transgene is fused to *dsRED* (*Pnlp-14::GCaMP6::SL2::dsRED*). The SL2 leader splices GCaMP and dsRED to give polycistronic expression. Since dsRED is co-expressed with GCaMP and is not affected by the changes in Ca^{2+} concentration, it is used as a reference to account for any

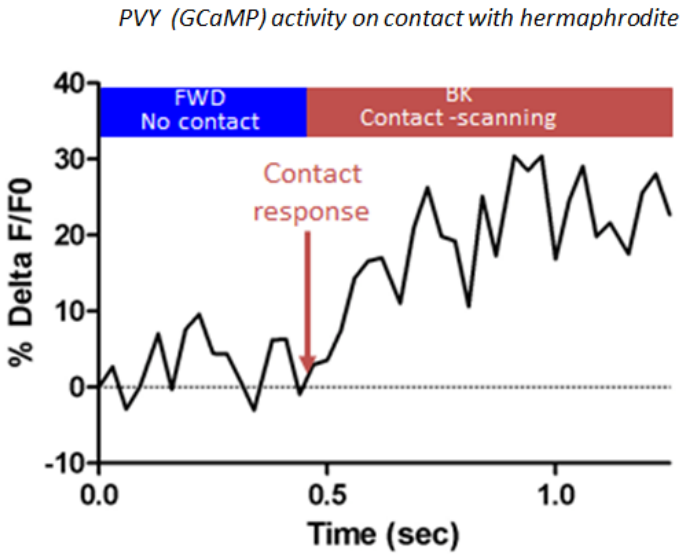

Figure 3.6 PVY is essential for contact response and maintaining backward movement during mating.
Representative trace of Ca^{2+} transients in PVY on contact with the hermaphrodite. All the males tested carry transgene *Pnlp-14::GCaMP6::SL2::dsRED*. The Y-axis is %ΔF/F0 and X-axis is time in seconds. Shaded area represents mating response on tail contact followed by backing.

changes in the GCaMP intensity due to movement of the cell, in or out of the focal plane. For the assays, one-day old transgenic virgin males were put on a mating lawn with 8-10 adult heat-treated *Phsp-16*:*egl-2(n693gf)cDNA; Punc-103E:mDsRed* hermaphrodites. *egl-2* encodes for a K^+ channel and heat shocking *egl-2(gf)* worms results in complete paralysis. *Punc-103D::dsRED* marks the vulva muscles and making it possible to visualize the vulva region under fluorescent conditions. The mating behavior was recorded using the Dual View Simultaneous Imaging system, which allows the dsRED and GCaMP fluorescence to be visualized simultaneously in separate channels. GCaMP fluorescent changes can be quantified relative to changes in dsRED. The changes in the fluorescence are plotted as % ΔF/F0 against time. ΔF is the difference in fluorescent intensity as compared to the baseline (F0 – fluorescent intensity of dsRED at time 0). Plotting ΔF/F0 corrects the expression level for GCaMP with respect to dsRED.

I focused on PVY activity because results from my previous experiments suggested that PVY plays a major role in vulva search locomotion. GCaMP fluorescent intensity increased in PVY within half a second of contact and stayed up while the male is backed on the hermaphrodite (Fig. 3.6). However, the increase in activity was graded as the changed with the speed modulations. This data along with the results of the optogenetic and ablation studies, confirms that PVY plays an essential role in inducing and maintaining backing during mating.

PVY uses cholinergic transmission to stimulate AVA

To investigate which neurotransmitter molecules and receptors are involved in the PVY/PVX induced reversals, we studied expression patterns of reporters for various neurotransmitter pathway genes. In addition to NLP-14, PVY and PVX expressed cholinergic marker UNC-17::GFP (Fig. 3.7A; Sherlekar et al., 2013). The *unc-17* gene encodes the synaptic vesicle acetylcholine transporter, required for loading acetylcholine into synaptic vesicles (Alfonso et al., 1993, Lickteig et al., 2001, Zhu et al., 2001). PVY/PVX might be using acetylcholine as a fast acting neurotransmitter to control male locomotion during mating. UNC-17 is widely expressed in *C. elegans* nervous system (Alfonso et al., 1993, Garcia et al., 2001) and *unc-17* mutants are extremely uncoordinated in locomotion (Brenner, 1974). Thus, using *unc-17* mutants to test whether cholinergic signaling is relevant to PVY/PVX induced backing will be uninformative. One way to overcome this issue is to use cholinergic receptors (*acr*) gene mutants, as teir site of action are more limited and defects less pleiotropic. First we determined which receptors are expressed in AVA. AVA interneurons express alpha nicotinic acetylcholine receptor subunits *acr-15* and *acr-16* (Jones and Sattelle, 2004, Feng et al., 2006), DEG-3 type acetylcholine receptor *acr-18* (Fig. 3.7B; Sherlekar et al., 2013), and a non-alpha receptor subunit *unc-29* (Fig. 3.7C; Sherlekar et al., 2013).

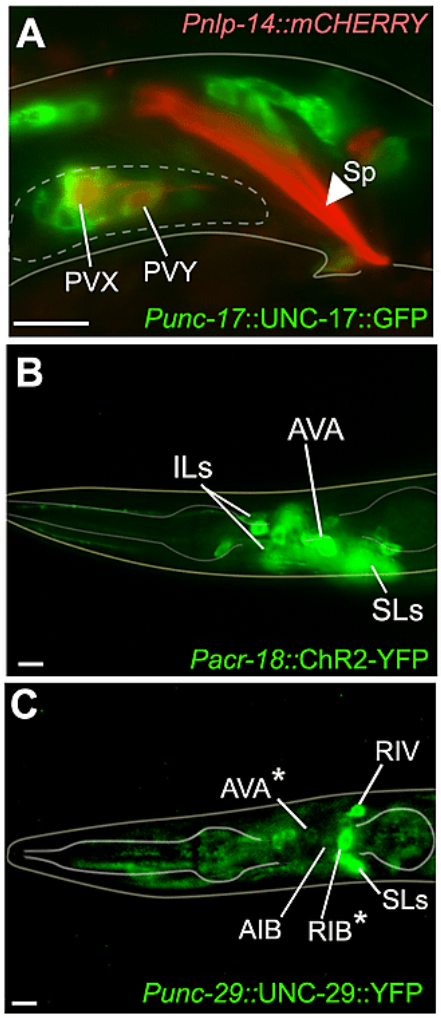

Figure 3.7 PVY and PVX are cholinergic and backward command interneurons, AVA express multiple cholinergic receptor genes.
A. Fluorescent micrograph of adult male tail (lateral view, posterior to right) showing co-expression of NLP-14::mCHERRY (PVY/PVX marker) and UNC-17::GFP (cholinergic marker). PVY, PVX are located in pre-anal ganglion (PAG) indicated by the dotted line. SP indicates spicule auto-fluorescence.
B. Fluorescent micrograph of L3 male head showing expression of *Pacr-18::ChR2-YFP* transgene in AVA.
C. Fluorescent micrograph of L3 male head expression of a full-length *unc-29* translational reporter. * indicates cells which co-express *unc-29* and *acr-16* transgene. Scale bar indicates 10 µm.
(taken from Sherlekar et al., 2013)

To test whether any of these receptors are required for PVY/PVX-to-AVA cholinergic transmission, PVY/PVX were artificially activated in various cholinergic receptor mutant backgrounds, using ChR2. *acr-18* single mutants showed a significant reduction in PVY/PVX induced reversals where only 28% males were able to back while 56% males kept moving forward and 16% paused (Fig. 3.8; Sherlekar et al., 2013). This suggests that *acr-18* activity is partially required to induce backing. *acr-18* is expressed in many sex-shared and male-specific neurons. To test if *acr-18* activity is required specifically in AVA, we tested whether restoring *acr-18* wild type function to AVA restores backing response. *acr-18* was rescued by placing the wild type *acr-18* transgene under the control of the *nmr-1* promoter, which is expressed in backward command interneurons AVA, AVD, AVE and few other neurons. The presence of wild type *acr-18* transgene was confirmed by the presence of co-injection marker mCHERRY, which was placed under the control of the *flp-18* promoter, also expressed in AVA. *acr-18* mutant males expressing *acr-18* wild type transgene were indeed more efficient in PVY/PVX induced reversals. The reversal response was rescued in 76% males as compared to reversal response in 28% *acr-18* mutant males (Fig. 3.8; Sherlekar et al., 2013). Together these results support the hypothesis that cholinergic receptor subunit *acr-18* acts in the backward command interneurons (likely AVA) to mediate PVY/PVX-to-AVA cholinergic transmission.

acr-18 receptor subunit activity accounts for part of PVY/PVX induced reversals, suggesting contributions from other cholinergic receptor subunits. I activated PVY/PVX in *acr-15* and *acr-16* single mutants which are expressed in backward command

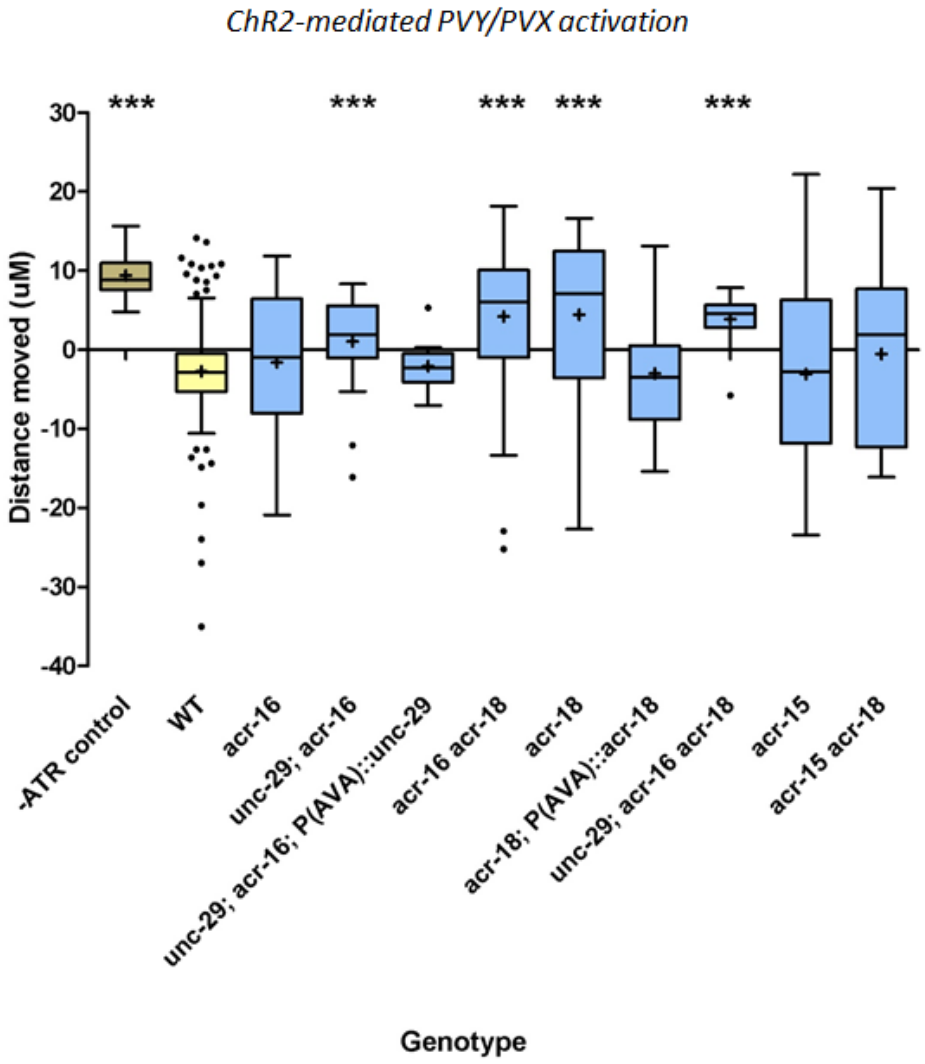

Figure 3.8 PVY/PVX modulates AVA interneurons via cholinergic signaling.
Cholinergic receptor mutants show reduced backing response on artificial activation of PVY/PVX using ChR2. All the animals carry the transgene *Pnlp-14(PVY,PVX)::ChR2-YFP*. See legend for Fig. 3.3. The X-axis indicates genotype. Except for "-ATR Males" treatment, all animals tested were grown on ATR supplemented OP50 *E. coli* (food). All males with *unc-29* mutation in the background carry *rgIs1* transgene which specifically rescues the mutation in body wall muscles.
A box-plot representation of the data shown, with median and mean values indicated by a line and a plus "+" sign, respectively. Statistical comparisons were made using ranksum test for differences in median. Significance, ***p <0.001

interneurons, AVA. The backing response reduced a little but was not significantly different from the wild type (Fig. 3.8; Sherlekar et al., 2013). To eliminate the possible redundant activity of *acr-18* subunit, I also activated PVY/PVX in *acr-15 acr-18 and acr-16 acr-18* double mutant background. *acr-15 acr-18* double mutant reversal response was not significantly different from the wild type and *acr-16 acr-18* backing response was similar to the *acr-18* single mutants (Fig. 3.8; Sherlekar et al., 2013).

AVA interneurons also express a non-alpha receptor subunit *unc-29*. It has been shown in previous studies that *unc-29* is functionally redundant with *acr-16* in body wall muscles used for locomotion and in some male-specific muscles involved in spicule insertion behavior (Francis et al., 2005, Liu et al., 2011). Mating analyses of *unc-29; acr-16* double mutants done in our lab, revealed slow scanning behavior similar to PVY-ablated males. In these animals, *unc-29* function was rescued in body wall muscles as the absence of both *unc-29* and *acr-16* from muscle results in paralysis, thus precluding mating studies (Ballivet et al., 1996, Francis et al., 2005, Touroutine et al., 2005). Specifically, a wild type *unc-29* cDNA was placed under the control of the *acr-8* promoter (expressed in body wall muscles and few VNC neurons) generating the transgene *rgIs1 [Pacr-8::unc-29cDNA::SL2::GFP]* (Ballivet et al., 1996, Brockie et al., 2001, Francis et al., 2005, Touroutine et al., 2005, Liu et al., 2011). To further test the contribution of *unc-29* and *acr-16* to PVY/PVX induced reversals, I activated PVY/PVX in *unc-29; acr-16* double mutant males carrying *rgIs1*. Only 31% of these animals showed backing response (Fig. 3.8; Sherlekar et al., 2013). This indicates *unc-29* and *acr-16* together play an important role in PVY/PVX induced backing behavior. To

further confirm that the site of action for *unc-29* is in AVA interneurons, we rescued *unc-29* specifically in AVA interneurons by placing it under the control of the *nmr-1* promoter. Eighty six percent of the rescued males reversed (Fig. 3.8, Sherlekar et al., 2013), confirming that the UNC-29 receptor subunit plays a role in PVY/PVX-to-AVA cholinergic transmission.

To further test whether the three cholinergic receptor subunits *acr-18, acr-16* and *unc-29* are sufficient for the PVY/PVX induced activation of AVA interneurons, I activated PVY/PVX in *unc-29; acr-16 acr-18* triple mutant background. Indeed, only 4% of the *unc-29; acr-16 acr-18* triple mutant males were able to reverse on PVY/PVX activation (Fig. 3.8; Sherlekar et al., 2013). Taken together, these data support a model in which PVY/PVX use cholinergic signaling to modulate the activity of AVA interneurons during mating and this transmission is mediated by cholinergic receptor subunits ACR-18, ACR-16 and UNC-29.

Rescue experiments with the cholinergic mutants were performed by Robyn Lints.

Cholinergic receptor mutants exhibit defective movement during mating

Mutations in *acr-18, unc-29* and *acr-16* significantly compromised reversal efficiency in the context of ChR2 assays. I next examined whether these mutations correspondingly caused vulva search defects in the context of mating. In spite of showing 60% reduction in reversals in the ChR2 PVY/PVX assays, *acr-18* mutant males exhibited wild type mating behavior except for some difficulty in contact response (Fig. 3.9A; Sherlekar et al., 2013). Similarly, *unc-29; acr-16* double mutant showed varied

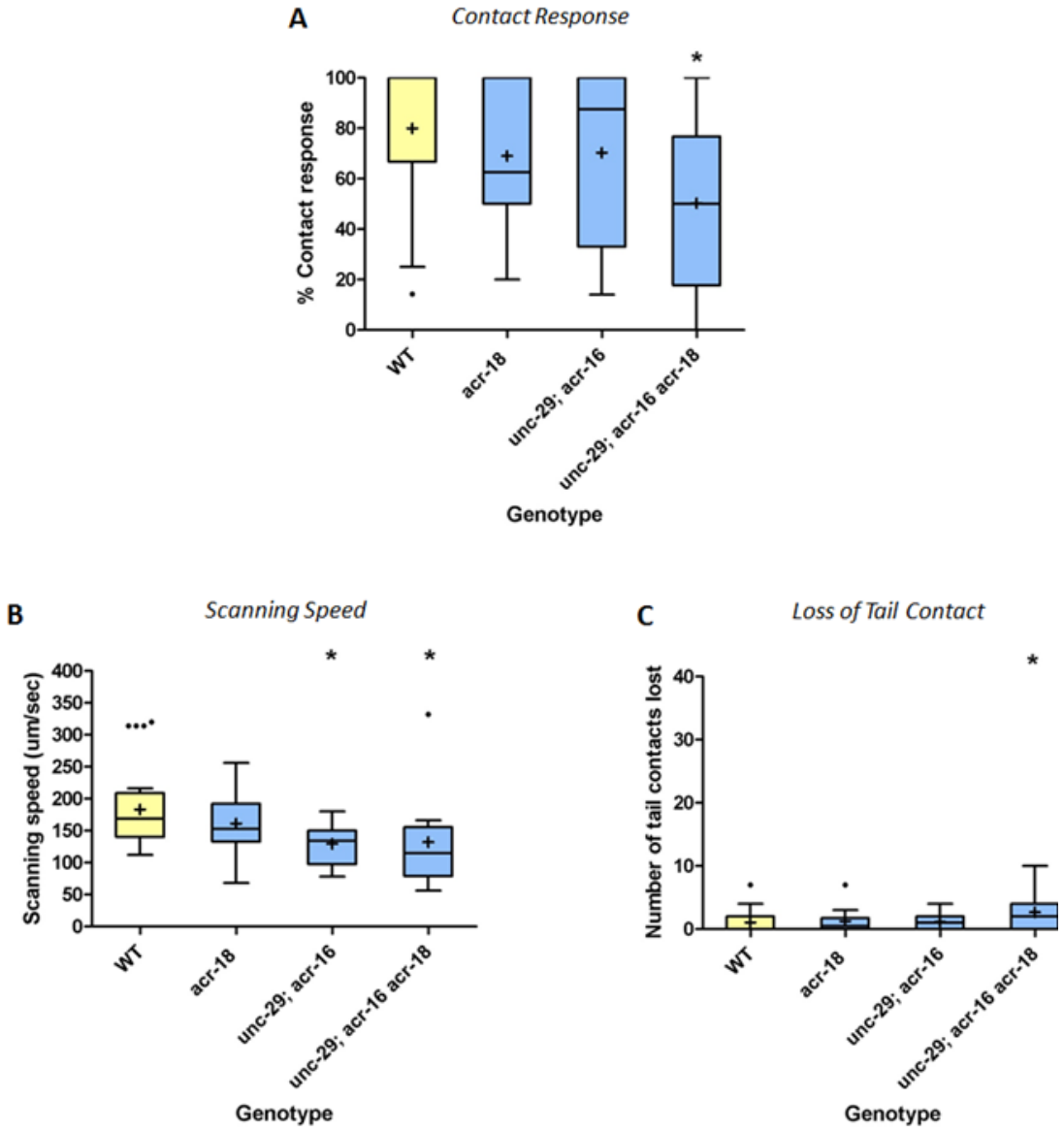

Figure 3.9 Cholinergic receptors play a functionally redundant role in mating locomotion.

A, B, C. The impact of cholinergic receptor mutations on the initiation and efficient maintenance of vulva search behavior. See legend for Fig. 3.1 and Fig. 3.8.

A box-plot representation of the data shown, with median and mean values indicated by a line and a plus "+" sign, respectively. n for each genotype: Mock=36; *acr-18*=12, *unc-29; acr-16*=14; *unc-29; acr-16 acr-18*=12. Statistical comparisons were made using ranksum test for differences in median. Significance, *p <0.05

defects in contact response though not significantly different from wild type. However, these double mutants exhibited a slower scanning speed (150µm/sec) than the wild type (Fig. 3.9B; Sherlekar et al., 2013). A possible reason for these relatively mild defects is that *unc-29; acr-16* and *acr-18* are partially redundant. I therefor examined *unc-29; acr-16 acr-18* triple mutant mating behavior. The triple mutant males were defective in all aspects of the vulva search behavior scored and magnitude of these defects was similar to PVY and PVX-ablated males (Fig. 3.2B, Fig. 3.9B; Sherlekar et al., 2013). These data strongly support partially redundant roles for acetylcholine receptor subunits *unc-29, acr-16* and *acr-18* most likely in PVY/PVX-to-AVA transmission. Also, like in PVY and PVX-ablated males, *unc-29; acr-16 acr-18* triple mutant males can still back slowly. This suggests the presence of a parallel pathway which may be able to compensate the loss of the main backing pathway in its absence, making mating locomotory circuits functionally robust.

EF neurons are acting parallel or upstream to the PVY-controlled backing circuit

The above results suggest that rays may use more than one pathway to regulate backward movement. The sensory rays have many post-synaptic targets which could be possible candidates for such parallel pathways. However, sensory rays have the highest number of inputs on male-specific interneurons of pre-anal ganglion, EF1-3 (Jarrell et al., 2012). EF neurons form reciprocal connections with most of the male-specific neurons and are unique in their morphology, being the only male-specific tail neurons which have processes running all the way up to the nerve ring of the worm head.

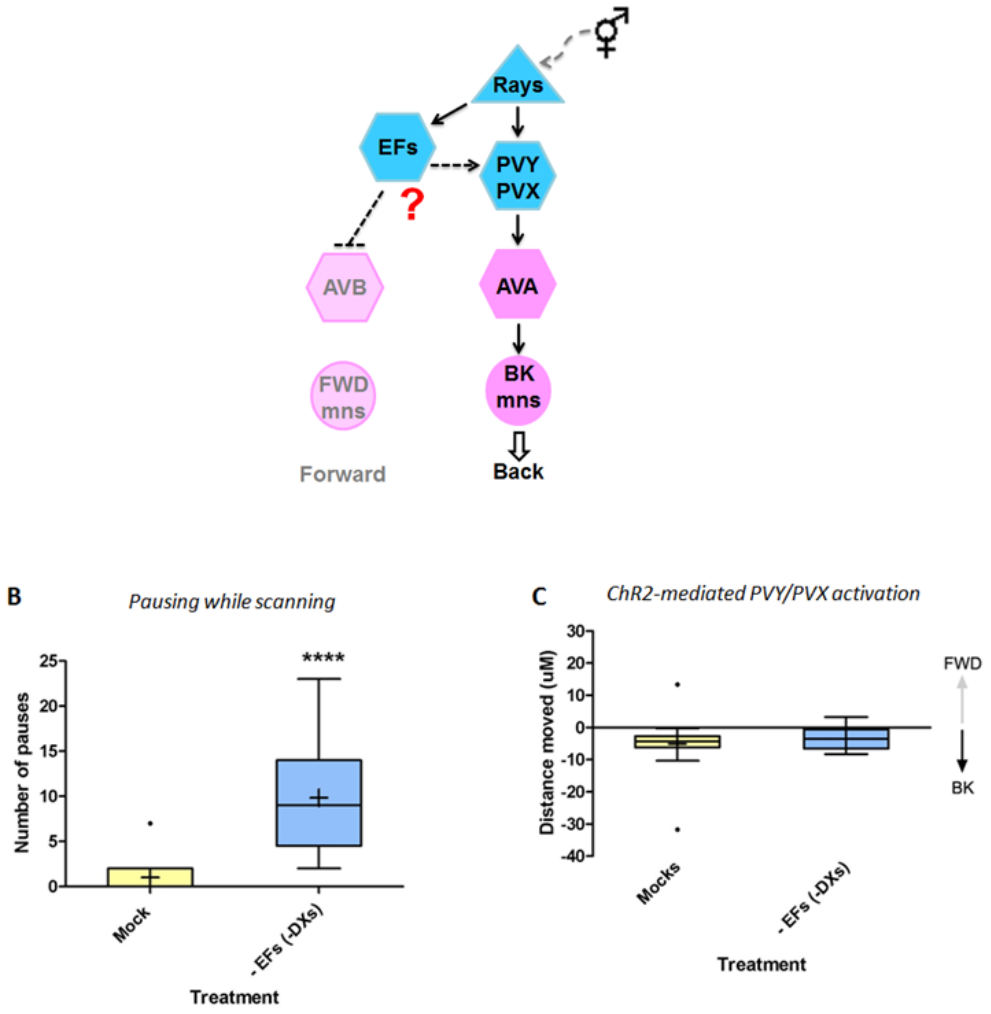

Figure 3.10 EF interneurons are required for continuous backing.
A. Wiring diagram showing EF interneurons connectivity to the locomotory circuit (based on male tail connectivity; (Jarrell et al., 2012). EF interneurons are presynaptic to forward command interneurons, AVA and male-specific interneurons PVY and PVX.
For key, refer legend for Fig. 3.1.
B. The impact of F and U ablations on the maintenance of vulva search behavior. F and U ablations eliminate EF and DX neurons. The number of pauses indicates discontinuous backing. To eliminate pauses induced on vulva detection, only pauses on non-vulva side of the hermaphrodite were quantified.
C. Artificial activation of PVY/PVX in EF ablated males did not affect the reversal response. All the animals carry the transgene *Pnlp-14(PVY,PVX)::ChR2-YFP*.
Statistical comparisons were made using ranksum test for differences in median. Significance, ***p <0.001

Interestingly, one of the major post-synaptic target of EFs are forward command interneurons, AVB(L/R) (S. W. Emmons, personal communication). Also, EFs are pre-synaptic targets of PVY and PVX (Fig. 3.10B). To test if EF interneurons play a role in locomotory control, I laser ablated the precursor cells F and U at L1 stage eliminating EF and their lineal sisters, DX interneurons. Killing the precursors was necessary because there are no known reporter genes which can be used to mark EF interneurons. Also, the number of EF cells varies from individual to individual (Sulston et al., 1980). The precise F and U pattern at L1 is known, making it easier to identify and kill the cells. The ablated and mock animals were grown to L4 stage and then the males were isolated on fresh plates and allowed to mature into adults. Their mating behavior was recorded and analyzed. The EF-ablated males were defective in mating, though their defects differ from PVY-ablated males. Specifically, backing was erratic such that EF-ablated males paused frequently during scanning. Males typically stop at the vulva, to prod at the vulva slit for spicule insertion and insemination. The median number of pauses on the non-vulva side was 9 in EF-ablated males, as compared to a median of 0 in mocks (Fig. 3.10B). The discontinuous backing in EF-ablated animals indeed suggests that EF interneurons activity contributes to backing.

The connectivity suggests that EFs act by suppressing the activity of forward command interneurons AVB (Fig. 3.10A; Jarrell et al., 2012). Also, DXs do not have connections to command interneurons, suggesting that they are not contributing to the locomotory circuit (Jarrell et al., 2012). In the absence of EFs, AVB activity might not be completely inhibited and this may interfere with backward pathway activity. Another

possibility can be EFs are reinforcing the PVY and PVX activity to maintain backing through their stimulation by the rays. To test whether EFs modulate PVY/PVX activity, I activated PVY/PVX in males lacking EFs. The EF-ablated males showed wild type PVY/PVX induced reversals (Fig. 3.10C), suggesting that the EF interneurons either act parallel or upstream of PVY and PVX.

In conclusion, these results support a simple backing circuit model for vulva search. The sensory rays target the male-specific interneurons in the PAG to induce backward locomotion. The critical targets are interneurons PVY and PVX which initiate and maintain backing by modulating the sex-shared backward locomotory circuit. Also, EF interneurons may contribute by suppressing the forward command interneurons, AVB. Together, male-specific interneurons PVY, PVX and EFs override the forward directional bias and induce backing. Further ablation studies are required to kill PVY, PVX and EFs together and determine the combined functions of these interneurons in the vulva search behavior.

CHAPTER IV

THE NEURAL MECHANISMS CONTROLLING PAUSING AT THE VULVA

Hook neuron HOA does not contribute significantly to induce pausing at the vulva

During mating, the male stops scanning upon encountering the vulva and starts prodding his spicules in an attempt to breach the vulva for insemination. The ventral side of the male tail has male-specific sensory neurons; sensory rays, hook neurons and post cloacal sensilla (p.c.s.) neurons (Fig. 1.2A). Previous ablation studies show that males lacking hook neurons are unable to detect the vulva and continue scanning without stopping (Liu and Sternberg, 1995). How hook neurons inhibit movement is completely unknown. The hook comprises two neurons, HOA and HOB (Sulston et al., 1980). In the wiring diagram, it is possible to trace pathways from the hook neurons to the forward and backward command cells some involving PVY/PVX and EFs (Jarrell et al., 2012). These putative pathways suggest that hook neurons could either activate the forward command interneurons (Pathway 1 in Fig. 4.1A) to counteract the backward pathway activity or alternatively inhibit the backward circuit (Pathway 2 in Fig. 4.1A) to induce pausing (Jarrell et al., 2012). The third possibility is that hook neurons may use both mechanisms to achieve pausing.

In the circuits for possibility (1), hook neuron activation of forward command interneurons (Pathway 1 in Fig. 4.1A). HOA connects indirectly to the forward command interneurons, AVB(L/R) via AVG. AVG is a single interneuron with the cell body located in the retrovesicular ganglion (RVG) of the head and its process extends

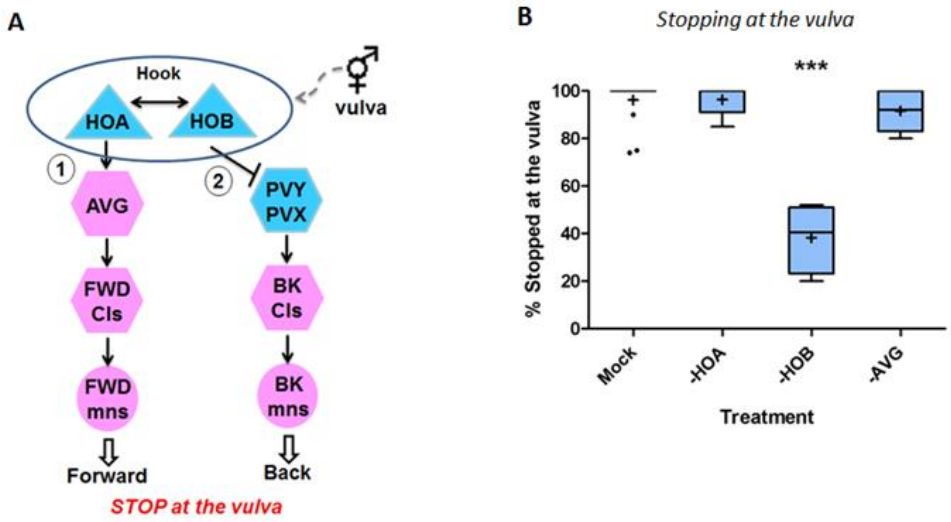

Figure 4.1 Male-specific hook neurons induce pausing on vulva detection.
A. A putative neural circuit for hook neuron regulation of backward circuit (based on (Jarrell et al., 2012).
Key: Sex-shared cells (pink), male-specific (blue), sensory neurons (triangles), interneurons (hexagon), motor neurons (circles). Stimulus (dotted arrow), stimulatory chemical transmission (arrows), inhibitory chemical transmission (T-bar), possible cross-talk (double-headed arow).
B. The impact of cell-specific ablations on the efficiency of the male to stop on vulva detection. The Y-axis indicates % Stopped at the vulva = 100 x [the number of times the male stopped at the vulva/the number of times the ventral part of the male tail came in contact with the vulva]. The X-axis indicates Treatment.
A box-plot representation of the data shown, with median and mean values indicated by a line and a plus "+" sign, respectively. Statistical comparisons were made using ranksum test for differences in median. Significance, ***p <0.001

along the VNC to the PAG. AVG is sex-shared and is primarily used as a guidepost cell to define the right longitudinal tract of the ventral nerve cord (Wadsworth et al., 1996). In the male, but not hermaphrodite, AVG is connected to the AVB forward interneurons by gap junctions (Jarrell et al., 2012). To determine if this simple HOA-to-AVG-to-AVB circuit induces pausing (Pathway 1 in Fig. 4.1A), I eliminated HOA or AVG by laser

ablations and measured the ability of the male to stop at the vulva. To visualize HOA for ablations, I used transgenic males carrying *Peat-4::G-CaMP3::SL2::mDsRed*. To visualize AVG, I used *Pflp-7::GCaMP V6 medium-dsRED* transgenic males. To reduce the possibility of developmental compensation, HOA and AVG were ablated at L4 stage. The ablated males were allowed to recover and mature overnight, separate from hermaphrodites. The mock animals were treated similarly to the ablated animals except for the laser exposure (refer Experimental Procedures). Each male was assayed for their mating behavior by putting an individual one-day adult male with five one-day adult hermaphrodites on a mating lawn. The mating behavior was recorded for 15 minutes or until the male ejaculated. Each mating trial was analyzed for the number of times the male stopped at the vulva. Similar to mock males, males lacking HOA invariably detected the vulva and paused there (Fig 4.1B). My results are contradictory to results from a previous study which show that HOA-ablated males cannot stop at the vulva (Liu and Sternberg, 1995). A possible explanation for this is that collateral damage may have been more likely with the equipment used 20 years ago. Additionally, I used a fluorescent marker (dsRED) to identify HOA cell body and loss of marker expression may be a more sensitive indicator of cell death than morphological criteria (blebbing or cell body swelling). Eliminating AVG had no effect on the pausing behavior at the vulva. The AVG-ablated males missed the vulva 1 out of 10 times which was not significantly different from the mock males (Fig 4.1B). These results suggest that HOA and AVG might have a redundant role to HOB and its post-synaptic targets.

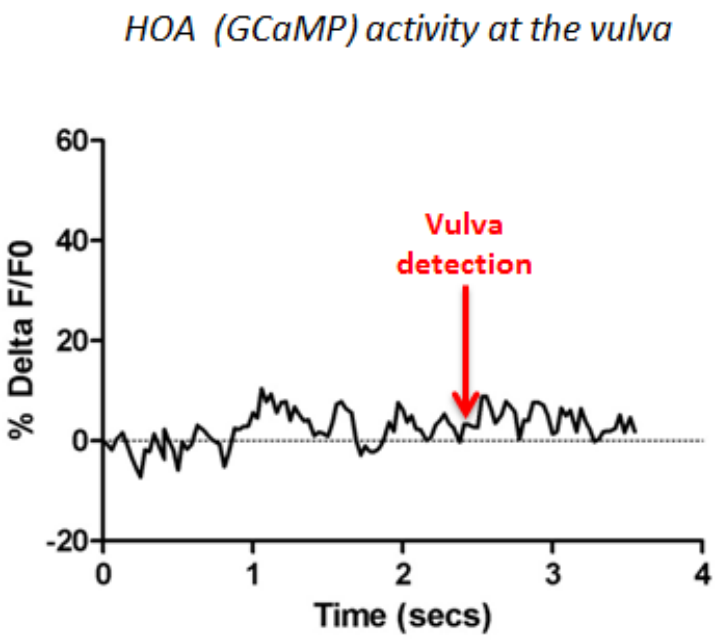

Figure 4.2 HOA activity does not change at the vulva.
Representative Ca^{2+} transients in HOA on vulva detection. All the males tested for HOB carry transgene *Ppkd-2::GCaMP V6 medium::SL2::dsRED.*

To further analyze the role of HOA, I studied HOA activity when the male was at the vulva using Ca^{2+} sensor GCaMP. All the males tested carried the transgene *Peat-4::G-CaMP3::SL2::mDsRed*. The *eat-4* promoter drives the expression of GCaMP and dsRED in HOA. The mating assays were performed by placing a one-day old adult, virgin *Peat-4::G-CaMP3::SL2::mDsRed* male with 8-10 paralyzed *Phsp-16:egl-2(n693gf)cDNA; Punc-103E:mDsRed* hermaphrodites (heat shocked for 2-3 hrs; refer Experimental Procedures). A Dual View Simultaneous Imaging system was used to record GCaMP and dsRED fluorescence intensities simultaneously in separate channels. dsRED fluorescence intensity was used as a baseline to measure the changes in GCaMP activity. The HOA GCaMP activity did not change as the male approached and

encountered the vulva (Fig. 4.2). This data suggests that HOA plays a minor role in stopping the male at the vulva, if any. The AVG GCaMP analyses showed similar results, namely no change in AVG activity was observed during the male's approach to the vulva or its detection (data not shown). Taken together, the ablation and GCaMP data for HOA and AVG indicates that HOA-to-AVG-to-AVB circuit does not play a significant role in promoting pausing at the vulva.

Investigation of hook neurotransmission mechanisms

Several neurotransmitter markers are known to be expressed in hook neurons, HOA and HOB. HOA is glutamatergic (evidenced by expression of *eat-4* marker). HOB expresses markers for neuromodulators NLP-8 (Nathoo et al., 2001) and FLP-5 (Kim and Lee, 2011). The receptors for NLP-8 and FLP-5 are not known and hence the post-synaptic targets cannot be tested. Recently HOB has been found to be positive for cholinergic marker (B. LeBoeuf and L. R. Garcia, personal communication).

To test the role of glutamate in stopping at the vulva, I tested mating behavior of *eat-4* mutant males (refer Experimental Procedures). *eat-4* gene encodes for vesicular glutamate transporter and mutant animals are defective in glutamate signaling (Lee et al., 1999). The *eat-4* mutant males had marginal defects in locating the vulva (Fig. 4.3), suggesting glutamate might have a minor role in vulva detection. The *eat-4* mutation affects all the glutamatergic neurons (78/302 neurons in the hermaphrodite and 20 additional tail neurons in males (Serrano-Saiz et al., 2013). A more specific way to investigate glutamate signaling is to evaluate mating behavior in various glutamate

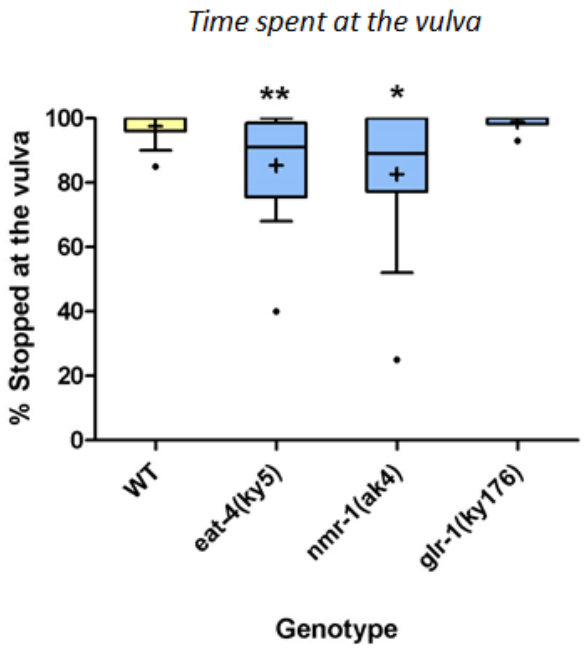

Figure 4.3 Glutamate signaling may have a minor role in pausing at the vulva. The impact of mutant males defective in glutamatergic signaling on the efficiency of the male to stop on vulva detection. The Y-axis indicates % Stopped at the vulva. Refer Fig. 4.1B legend for details. The X-axis indicates Genotype.
A box-plot representation of the data shown, with median and mean values indicated by a line and a plus "+" sign, respectively. Statistical comparisons were made using ranksum test for differences in median. Significance, *p <0.05, **p <0.01

receptor mutants. If glutamate is required then receptor mutants should phenocopy the *eat-4* mutant defects in vulva detection. NMDA-type ionotropic glutamate receptor subunit, NMR-1 is expressed in backward command interneurons and AVG. AMPA-type ionotropic glutamate receptor subunit, GLR-1 is expressed in both the backward and the forward command interneurons. In addition these neurons, NMR-1 and GLR-1 are expressed in many other sex-shared and male-specific neurons of the tail (A. Sherlekar unpublished; Brockie et al., 2001). I tested mating behavior of *nmr-1* and *glr-1* mutant males. The *glr-1* mutant males were similar to wild type males. The *nmr-1*

mutant males phenocopied *eat-4* mutant males in having amarginal defect in vulva detection (Fig. 4.3). These data suggests that glutamate plays a minor role in inducing pausing at the vulva. In context of HOB, further analyses of cholinergic receptor mutants should reveal the contribution of cholinergic signaling to this behavior.

HOB plays a major role to induce pausing at the vulva

The next step was to evaluate possibility (2) where the backward circuit is inhibited to induce pausing (Pathway 2 in Fig. 4.1A). Unlike HOA, the sensory endings of HOB are exposed to the external environment, bringing HOB in direct contact with the hermaphrodite surface during the vulva search (Sulston et al., 1980). To test whether HOB is important for pausing at the vulva, I laser ablated this neuron in *Ppkd-2::GFP* L4 males, where the cell body is marked. One-day old virgin, adult HOB-ablated and mock males were tested for their mating behavior. The males lacking HOB showed a significant reduction in their ability to detect the vulva. In HOB-ablated males, pausing at the vulva was reduced to 40% as compared to 100% in mock male. This indicates that HOB is required for stopping at the vulva (Fig 4.1B) and is consistent with the previous HOB ablation studies (Liu and Sternberg, 1995). It is also consistent with the results of mutant analyses on the three genes expressed in HOB, *lov-1*, *pkd-2* and *egl-46* mutants (Barr and Sternberg, 1999, Yu et al., 2003). EGL-46 is a transcriptional factor that regulates the expression LOV-1 and PKD-2 (Yu et al., 2003). Thus, loss of HOB function either by ablation or genetic mutation disrupts the male's ability stop at the vulva.

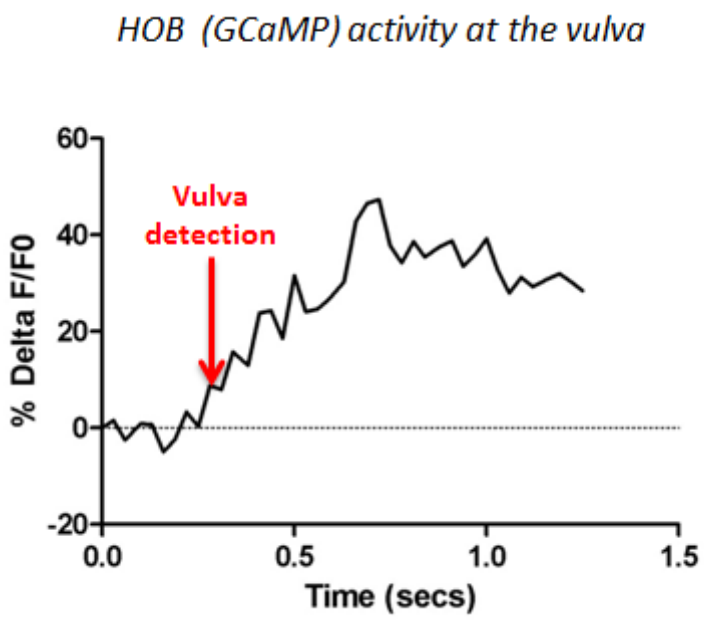

Figure 4.4 HOB is essential for stopping at the vulva.
Representative Ca^{2+} transients in HOB on vulva detection. All the males tested for HOA carry transgene *Peat-4::G-CaMP3::SL2::mDsRed.* The Y-axis is %ΔF/F0 and X-axis is time in seconds.

The HOB-ablation data was further supported the GCaMP assays performed to assess HOB activity during mating. To visualize HOB activity, I used a strain carrying *Ppkd-2::GCaMP V6 medium::SL2::dsRED* transgene. GCaMP and dsRED were simultaneously expressed in HOB under the control of *pkd-2* promoter. The mating assays were performed and the HOB activity was analyzed in the same way as described for the HOA GCaMP assays. Upon vulva detection, the GCaMP activity in HOB increased several fold (Fig. 4.4). These data, along with the ablation data confirm that HOB activity is needed for vulva detection. However, elimination of HOB does not entirely disrupt stopping at the vulva. One possibility is that HOA may compensate in

absence of HOB. The GCaMP assays show that HOA activity does not change at the vulva. Visualizing HOA activity in the absence of HOB would be one way of testing this hypothesis.

PVY/PVX and EFs are essential for staying at the vulva

To explore the possibility that backward circuit components contribute to pausing at the vulva, I analyzed PVY/PVX- and EF-ablated males for their ability to stop a t the vulva. An unexpected finding of this study was that PVY/PVX- and EF-ablated males spent significantly less amount of time at the vulva during the mating trial. The mock animals spent a median time of 300secs at the vulva in an attempt to insert their spicules. The median time spent at the vulva by PVY/PVX-ablated males was 40secs and by EF-ablated males was 60secs (Fig. 4.5B). These results suggest that the backward circuit is the backward circuit might be contributing to either maintaining the tail over the vulva or to spicule insertion (Fig 4.5A).

To further investigate the contribution of these cells during spicule insertion attempts, I studied PVY activity using GCaMP when the male was at the vulva. The GCaMP assays were performed on *Pnlp-14::GCaMP V6 medium::SL2::dsRED* males as described in the previous sections (refer Experimental Procedures). There was a small decrease in the PVY activity on vulva detection (Fig. 4.6) consistent with the possibility that HOB is inhibiting PVY to induce pausing. Upon vulva detection, the male changes his tail posture, cupping the vulval mound slightly to maintain his position (Liu et al., 2011). This posture change is mediated by a major hook neuron target, the p.c.s.

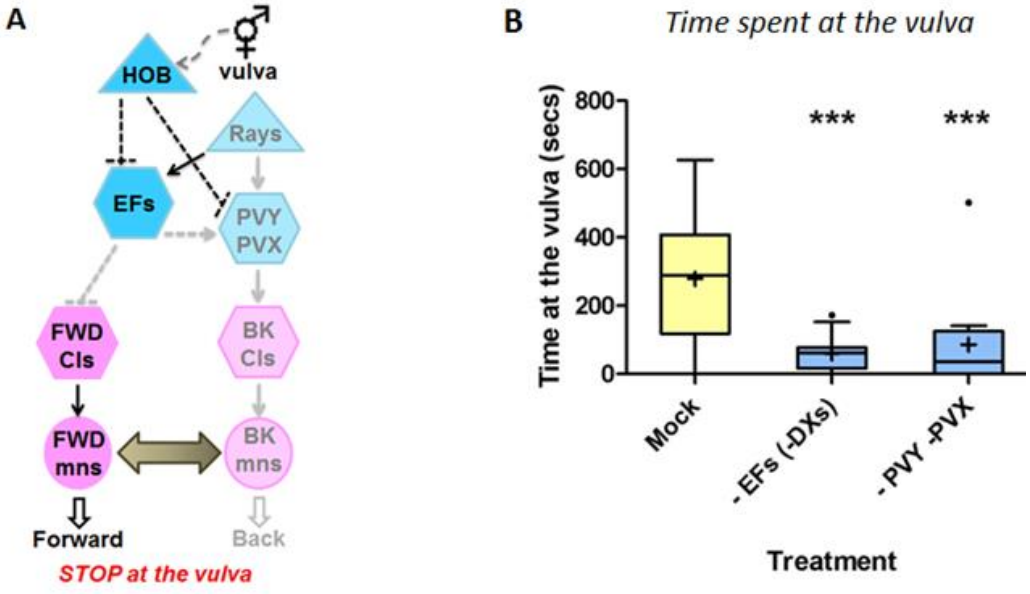

Figure 4.5 Male-specific interneurons controlling backing are also required for staying at the vulva.
A. Wiring diagram explaining the regulation of backward circuit components by hook neuron, HOB (based on Jarrell et al, 2012). Key: Refer Fig. 4.1A legend
B. Males lacking PVY/PVX or EFs cannot stay at the vulva. –PVY-PVX animals carry the transgene *Pnlp-14(PVY,PVX)::ChR2-YFP*. The Y-axis indicates the time spent at the vulva during a 15-minute mating trial or until the male ejaculated, whichever occurred first. The X-axis indicates Treatment. The controls are indicated by "Mock". A box-plot representation of the data shown, with median and mean values indicated by a line and a plus "+" sign, respectively. Statistical comparisons were made using ranksum test for differences in median. Significance, ***p <0.001

neurons. These neurons synapse with several tail muscles and likely promote their differential contractions through cholinergic transmission, which is the dominant transmitter of the p.c.s. neurons (Liu and Sternberg, 1995, Garcia et al., 2001, Liu et al., 2011).

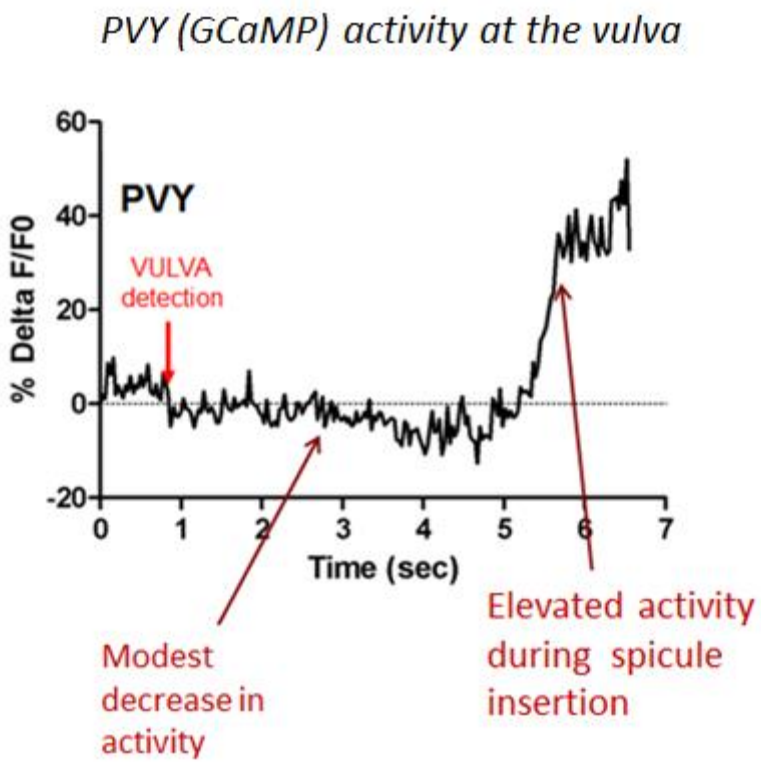

Figure 4.6 PVY activity is required during spicule insertion.
Representative Ca^{2+} transients in PVY at the vulva. In an attempt to breach the vulva, males prod their spicules increasing the PVY activity. All males tested carry transgene *Pnlp-14::GCaMP6::SL2::dsRED.* The Y-axis is %ΔF/F0 and X-axis is time in seconds.

PVY activity increases during the spicule insertion attempts (Fig. 4.6). *Pnlp-14::GCaMP V6 medium::SL2::dsRED* transgene is sometimes expressed in one or two cells of dorsorectal ganglion (DRG) in the posterior part. The increase in PVY-activity coincides with the increase in activity of the two DRG cells. Based on the location, these cells are possibly male-specific interneurons DVE or DVF. Interestingly, these cells form significant connections to the SPC neurons which control spicule prodding and

insertion. Moreover, PVY is electronically coupled to DVF. These results suggest an interesting possibility that PVY activity could contribute to spicule insertion.

In conclusion, these results suggest that hook neuron HOB induces pausing at the vulva and probably acts redundantly with HOA. Further studies are required to test the contribution of cholinergic signaling in HOB function. Additionally based on the neuropeptide repertoire of the hook neurons, there is an interesting possibility that the neuromodulators released by the hook neurons act to alter the circuit affiliations of the backing circuit components. Upon vulva detection, the hook neuromodulator action could be uncoupling PVY from AVA, to associate with the DRG cells of the spicule insertion circuit.

CHAPTER V

SUMMARY OF EXPERIMENTS AND DISCUSSION

The rays exploit the sex-shared locomotory system to control vulva search locomotion

In this study I used an integrative approach by combining connectivity data, genetics, optogenetics and laser ablations to delineate the neural circuits controlling male locomotion during mating. My studies reveal that the rays exploit the sex-shared locomotory system and control it via multiple upstream inputs. The rays exert their effect via two male-specific pathways that converge on the command cells of the system. Interneurons PVY and PVX define the major control pathway and EF1-3 interneurons define a second, possibly minor pathway (Fig. 5.1; Sherlekar et al., 2013). Each pathway converges on the control centers of the sex-shared locomotory system: PVY and PVX target the AVA backward command interneurons and the EFs, the AVB forward. Our working model is that PVY activation of AVA promotes backward movement, while EF inhibition of AVB enhances the efficiency of reversal or confers its fine control. The use of parallel pathways would explain the robustness and accuracy of ray-controlled movement during mating. The model for PVY/PVX involvement is supported by multiple and independent lines of data (cell-specific ablation, optogenetics and mutant analyses). The involvement of EFs is supported by ablation data only and will require further experimentation once promoters expressed in EFs are identified and

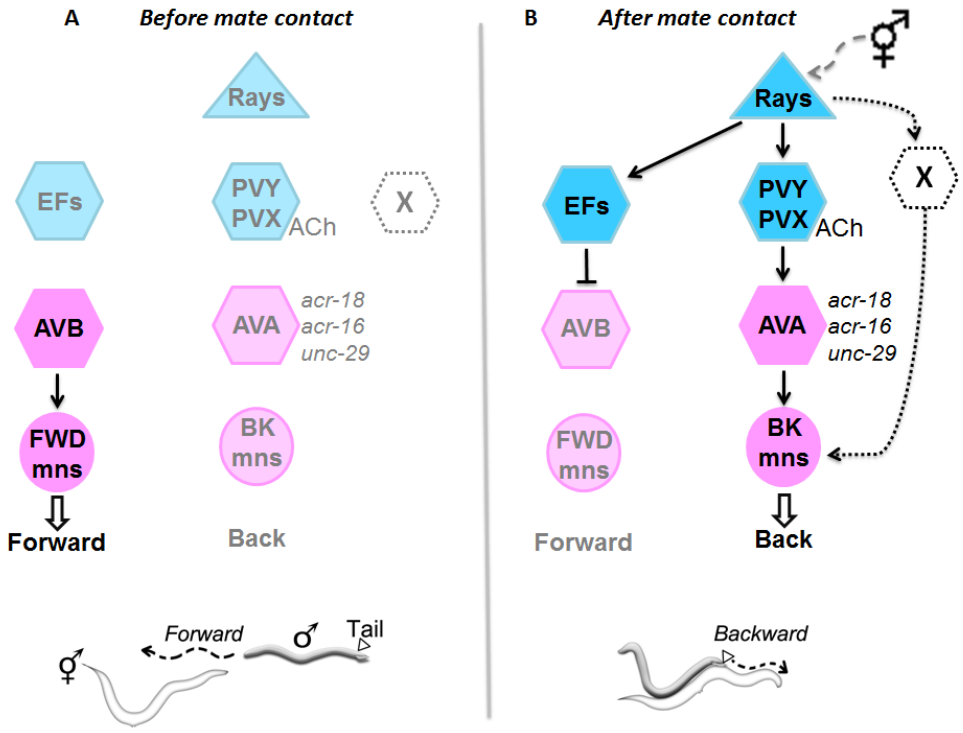

Figure 5.1 A circuit model for backward locomotion during male mating behavior.

A, B. A circuit model for mate-induced locomotory changes in male. The different shapes indicate the type of neurons: Sensory neurons (triangles), interneurons (hexagon), motor neurons (circles). Color indicates the sex-specficity: male-specific neurons (blue) and sex-shared neurons (pink). The arrows indicate activation of the post-synaptic targets and T-bar indicates inhibition of post-synaptic target. Color intensity indicates cell activity (Dark color=high activity, faint color=low activity).

A. In absence of mate contact, the male moves with a forward directional bias because of higher activity of forward command interneurons, AVB and B-motor neurons (FWD mns). The forward bias is an intrinsic property of the sex-shared locomotory circuit (Kawano et al., 2011) and represents the exploratory behavior of the worm.

B. On contact with the mate, ray neurons initiate contact response and induce backing by activating male-specific interneurons, PVY and PVX. The cholinergic PVY, PVX activate backward command interneurons AVA via acetylcholine receptor subunits ACR-18, ACR-16 and UNC-29. AVA interneurons further activate A-motor neurons (BK mns) to produce backward directional movement. Ray-neurons send parallel inputs to male-specific EF interneurons which possible promote backing by inhibiting forward command interneurons, AVB. "X" indicates the PVY/PVX-independent pathway used by ray neurons to promote backing.

it neurotransmitter fate determined through comprehensive gene analyses. The wiring diagram also indicates other ray-to-locomotory system connections worthy of in future investigation. These include sex-shared interneurons PVN, AVF, AVJ and AVH ("X" in Fig. 5.1; Sulston and Horvitz, 1977, Sulston et al., 1980, Jarrell et al., 2012, Sherlekar et al., 2013).

PVY/PVX regulate the command cells using an atypical transmission mechanism

In hermaphrodite locomotion, directional change during spontaneous reversals and in response to stimuli is effected by glutamatergic regulation of command interneurons (Brockie and Maricq, Hart et al., 1995, Maricq et al., 1995, Chalasani et al., 2007, Ohnishi et al., 2011, Piggott et al., 2011). However, PVY/PVX-induced directional shift towards backing is mediated by cholinergic signaling. The cholinergic receptor subunits ACR-18, ACR-16 and UNC-29 mediate the increase in AVA activity to promote backing (Fig. 5.1; Sherlekar et al., 2013). A previous study has shown that command interneuron activity is regulated via AchRs in response to exogenous nicotine (Feng et al., 2006). My study is the first to show that command interneurons can be regulated by cholinergic inputs in the context of a natural *C. elegans* behavior. Why does the *C. elegans* male use this atypical mode of neurotransmission? First, its atypical use may confer response specificity. Second, it may make it easier to coordinate vulva search behavior with other mating sub-behaviors, as many appear to depend on cholinergic transmission (Garcia et al., 2001, Whittaker and Sternberg, 2009, Liu et al., 2011, Siehr et al., 2011). ..

Finally, the kinetics of cholinergic signaling may confer some speed advantage in processing as many other rapid response behaviors in invertebrates also depend on acetylcholine several. For example, cholinergic signaling mediates escape response in *Drosophila*, snails, crayfish and crickets (Miller et al., 1992b, Fayyazuddin et al., 2006, Palikhova et al., 2006, Yono and Aonuma, 2008).

The design of the *C. elegans* locomotory system itself may also enhance processing speed. The command interneurons in *C. elegans* are structurally analogous to the giant fibers, as in their processes run along the entire length of the worm and they form chemical and electrical connections with motor neurons and other neurons (White et al., 1986). In other invertebrate systems rapid response behaviors are often mediated by mediated by giant fibers. Greater giant fiber diameter decreases resistance and increases impulse conduction speed (Hartline and Colman, 2007). Also, like *C. elegans* command interneurons and their motor neurons targets, giant fibers are electrical coupled to their motor neurons. These neurochemical and organizational design features enable the worm to rapidly process the information and respond quickly.

Male-specific interneuron PVY acts as a decision-making interneuron to switch the directional bias to backward

A decision-making cell acts as a center for the integration for multiple sensory inputs and is necessary and sufficient to induce changes in the motor output. PVY fulfills all these criteria and can be considered a decision-making cell in the male locomotion. The artificial activation of PVY (using ChR2) is sufficient to induce backing. Males

lacking PVY are defective in scanning for the vulva. Also, inhibiting PVY (using NpHR) while the male is scanning the hermaphrodite stops backing, indicating that PVY is necessary for backing. The levels of PVY activity during mating (visualized with GCaMP) also correlated positively with the initiation and speed of backward locomotion arguing that its activity played a direct role in driving backward momentum. Consistent with the notion that decision-making interneurons are to centers for sensory integration, PVY receives input from multiple sensory pathways: the male-specific rays, p.c.s. and hook (required for mating) and the sex-shared phasmids (required for mate-search behavior (Jarrell et al., 2012)

Regulation of the backward circuit to induce pausing at the vulva

The male-specific hook and p.c.s. neurons detect general and precise location of the vulva respectively, to induce pausing (Liu and Sternberg, 1995). How does their action interface with that of PVY, the EF interneurons and the command interneurons? One possibility is that the hook induces pausing by stimulating the forward command cells, thus a balancing backward and forward pathway activity. In support of this possibility, we identified a pathway connecting HOA to AVB via a sex-shared interneuron AVG. To determine whether this HOA-AVG-AVB pathway contributed to pausing behavior at the vulva I assessed the impact of ablating HOA or AVG on vulval location. Surprisingly I found that neither ablation interfered with pausing at the vulva. Additionally, I saw no change in AVG or HOA activity (visualized with GCaMP) during vulva detection. Together these data suggest that HOA-AVG-AVB connectivity does

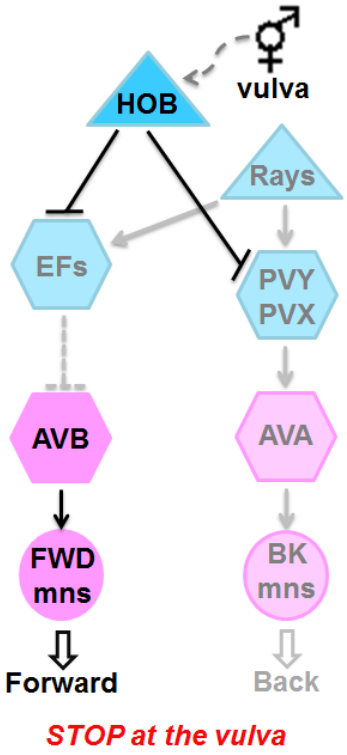

Figure 5.2 A possible circuit model for hook neuron induced locomotory changes at the vulva.
A putative circuit for how inputs from hook neuron, HOB alter the backward locomotory circuit activity to induce pausing at the vulva. The different shapes indicate the type of neurons: Sensory neurons (triangles), interneurons (hexagon), motor neurons (circles). Color indicates the sex-specficity: male-specific neurons (blue) and sex-shared neurons (pink). The arrows indicate activation of the post-synaptic targets and T-bar indicates inhibition of post-synaptic target. Color intensity indicates cell activity (Dark color = high activity, faint color = low activity).
Upon sensing vulva, hook neuron HOB might be inhibiting the PVY/PVX and EF interneurons to induce pausing by possibly using neuromodulators NLP-8 and FLP-5.

not contribute significantly to terminating the search and that the hook probably does not act by up regulating the forward locomotory pathway. This result also emphasizes that

though the wiring data may be compelling, the functional relevance of circuits can only be determined experimentally.

The alternative possibility is that hook neurons induce pausing by attenuating backward pathway interneurons by inhibiting PVY/PVX (Fig. 5.2). Consistent with this I observed that PVY decreases transiently with vulva detection. Other data suggests that PVY may this decrease in activity could be caused by HOB, rather than HOA activity. In contrast to HOA, HOB activity increases dramatically with vulva detection and HOB ablation significantly interferes with the success of this step. HOB has few connections with PVY but interestingly this neuron has recently been identified to be cholinergic (B. LeBoeuf and L. R. Garcia, personal communication). Further studies are required to verify this model and to identify the neurotransmission mechanism used by HOB to induce pausing at the vulva.

Another interesting observation made in my studies is that, paradoxically PVY and the EFs are also required to stay at the vulva. This may relate to the observation that, PVY activity (using GCaMP) increases at dramatically spicule insertion attempts. Two DRG cells, potentially DVE and DVF. also display an increase in activity suggesting that PVY, PVX and these DRG cells are acting in a circuit . Based on these data and the connectivity data, two possible models can be proposed to explain these observations. In the first model PVY/PVX induce backing in response to two different sensory systems, the rays and the hook and p.c.s. neurons. In the latter, changes in the tail posture with vulva location cause the backward force to be channeled into downward pressure that hold the tail over the vulva. In the second model, detection of the vulva causes

PVY/PVX to change their circuit affiliations so they become part of a spicule insertion circuit that also includes DVF and DVE. .

The mapping of male mating locomotory circuit has laid the groundwork to explore how decisions are made during mating. *C. elegans* male mating is a sequence of stereotyped steps in response to mate cues. For example, on reaching the hermaphrodite end the male curls his tail to go to the opposite side. Upon vulva location male starts prodding his spicules in an attempt to insert and inseminate. Depending on the hermaphrodite cues, how does a male decide which step to perform next? The male locomotory circuits have to be further investigated to answer how males decide to proceed with the sequential execution of the sub-behaviors of mating.

Altogether, my work on *C. elegans* male locomotory circuits revealed how sex-specific inputs control the existing sex-shared circuits by using distinct mechanisms to generate rapid behaviors. Also, how same circuit components are possibly used to control different motor outputs. Thus, studying smaller circuits in simple organisms will help us understand the underpinnings of more complex, larger circuits in higher organisms.

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