MALE MATING BEHAVIOR IN *Caenorhabditis elegans*

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1995

(Submitted May 24,1995)

Acknowledgments

A thesis is the product of not just one writer but the interactions of hundreds of people. However, I thank the following in particular. If I have forgotten or underappreciated anyone, I am sorry.

My mother and father, Diana H. and Kwei O. Liu, have my life long gratitude, not just for bringing me into this world, but also imbuing me with a reverence for learning. My parents left their own country and culture to pursue higher education in the United States. They have worked hard all of their lives to provide even better opportunities for me and my brother, Victor. This thesis is as much a culmination of their dreams as it is mine.

I thank David Weisblat for giving me my start in biology. He opened his lab to a wide-eyed undergrad (I was his first.), providing unlimited access to his time and equipment, and trusting me with an independent project. If not for him, I would most likely not be at Caltech.

For their time and interest, I thank the members of my thesis committee: David Anderson, Seymour Benzer, Mark Konishi, and Gilles Laurent. Their help has been especially invaluable to a graduate student trying to study neuro in a developmental lab.

None of this work would have been possible without the pioneering and much greater efforts of John Sulston and Jonathan Hodgkin. I don't know how many times they are cited here; I can't count that high. I also thank the members of the worm community in general, who have provided a cozy scientific environment in which to mature. I still question my decision to leave.

Chand Desai suggested that I start my analysis with the ablation studies, which ended up comprising the crux of my thesis. Christopher Schoenherr suggested I screen specifically for vulva location defective mutants when none turned up in the first screen. My revised screen found not only the missing vulva location mutants, but the "hermaphrodite specific Cods" as well. I like to think that I would have done these things even without their suggestions, but when?

Linda Huang has been my roommate and lab mate all these years. She has been my coconspirator in afternoon runs to McDonald's for Happy Meal toys or conversation over overly-sweet coffee drinks at Starbuck's. But more than that, she has been the tether to my graduate school wanderings. Always attentive and dependable, I've used her to gauge how far off track I was.

Many people have contributed to this body of work over the years: Leslie Barber, Will Boorstein, Helen Chamberlin, Ann Chen, and Yvonne Hajdu-Cronin. I would especially like to thank Yvonne for the hundreds of drudging hours that she's put into the Cod project. Chapter three would not exist without her.

Lastly, I thank Paul Sternberg, the best advisor. Period. He knew to push when I needed it. But more notably, he knew to leave me be when pushing wouldn't do any good. Throughout the years, he has provided support - financial, intellectual, and emotional. Paul has always been positive and enthusiastic about my (and everyone else's) work. Something for which he has been much teased... and much appreciated.

Abstract

Several key features about the nematode, *C. elegans*, make it a tractable model system for the analysis of behavior. *C. elegans* allows genetic analysis of behavior in an animal with few neurons and few connections between these neurons. Male mating behavior in *C. elegans* requires the coordination of several steps: response to contact with the hermaphrodite; turning around her head or tail; location of the vulva; insertion of the spicules into the vulva; and sperm transfer. I have chosen to study this relatively complex behavior in this simple system to begin to understand how complex behaviors are coordinated.

I have taken two approaches to the problem that take advantage of the unique characteristics of *C. elegans*. First, since all cells are reproducibly identifiable in this system, I lesioned individual structures and neurons to determine which neurons mediate mating behavior. By cell ablation, we have identified participating sensory neurons for each step of mating behavior. The sensory rays mediate response and turning. The neurons of the hook and post-cloacal sensillae mediate vulva location. Two pairs of the spicule neurons mediate spicule insertion, while another pair regulates sperm transfer. In addition, some inter and motor neurons have been identified which participate in these steps.

Second, I screened for mutants impaired in mating behavior. From the screen, we have isolated mutants defective in each step of the behavior. These results independently suggest that the different steps of mating behavior are independently mutable and therefore likely mediated by separable neuronal system. In addition, mutants were isolated that appear

to have hermaphrodite specific defects, indicating along with ablation results that a hermaphrodite signal is required for the initiation of sperm transfer.

In conclusion, I have found that the steps in mating behavior are to a large extent separable. Male mating behavior in *C. elegans* is not entirely innately controlled, but rather the initiation of each step and the integration between steps is highly regulated by sensory feedback.

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Chapter 1

Introduction

The general problem of "explaining" behavior

The first problem in this broad field is agreeing on what we mean by an explanation of behavior. Let us define a behavior as a characteristic response to external or internal stimuli. Also, let us define an "explanation" of behavior as a description of the physical processes that underlie the reception of the response and the execution of the behavior. At its simplest then, the explanation of that behavior would require identification of the triggering stimulus, the mode of sensory transduction into the animal, and the motor system which generates the response. This might account for a binary response, with little ability to process stimulus strength, no ability to modify response strength or choose between alternative responses.

However, most of what we call behavior is a complex sequence of these "stimulus-response" units that interact with one another to form a steady "stream." Thus, how an animal responds to a stimulus will influence the nature of the subsequent stimuli it encounters, which will in turn influence its behavior. Where "one behavior" starts and another begins is not always obvious.

To this sequence, add the effects of learning - that is, changes in the strength and nature of a response due to prior exposure to the same or similar stimuli. Then add the ability to choose between two motor outputs given the conflicting stimuli. The nervous system required to mediate this stream must be able to integrate several sensory inputs, compute a choice, and coordinate motor outputs to switch over (end one, begin the next, and determine the timing between the two), all linking several simple behavioral units into a more complex behavior. The task of describing the mechanisms

becomes daunting very rapidly. Yet even some of the simplest nervous systems are capable of doing these things.

There are several ways to simplify the problem. One could look at only one aspect of the behavior, for example sensory processing or motor coordination, in the hopes of linking them eventually. Alternatively, one can study a pared down model of a behavior seen in intact organisms, such as the study of LTP as a model for associative learning. Or one can pick a very simple organism and hope that what is learned there will be of some general use to understanding behavior. I have chosen the last alternative, studying male mating behavior in the nematode, *C. elegans.* The results of these studies are discussed in the following chapters. Here, I discuss the paradigms that have been developed in the pursuit of understanding.

In the pursuit of a physical explanation for behavior, the first thing one must do is to break up a stream of behavior into manageable, measurable units. Ideally, these divisions reflect divisions in the underlying processes that meditate them. However, where to draw the line is not always so straightforward. An example is the ongoing debate over whether classical and operant conditioning describe different or the same phenomena (Terrace, 1973). Having defined the behavioral unit of interest, there are two levels in reductionist biology at which one can try to determine the physical basis for the behavior - cellular and molecular. These levels are in no ways exclusive of each other. Proper understanding of either cannot occur without the other. As I will argue, the cellular and genetic (molecular) approaches to understanding behavior are complementary to each other, and both approaches are dependent upon proper identification and measurement of the behavioral problem of interest.

Approaches to the problem

Measurement of behavior

It was Darwin who set the groundwork for the use of animal models to study behavior (1871). Darwin astutely realized the implications of his theory of evolution. If the vast variation in all animals arose from a common source via the selection of heritable traits, their vastly varying behaviors must have also. Thus, "lower" animals must have behavioral traits in common with "higher" animals and can be studied to better understand the basis of behavior in animals such as ourselves. Based on this premise, and in reaction against the introspectionist school of psychology, Jennings (1906) and his colleagues advocated ignoring subjective, internal states and concentrating on overt behavior, which could be measured. During the late 19th to mid 20th centuries, the study of animal behavior split into two major camps: the ethologists, based predominantly in Europe, and the experimental psychologists, based predominantly in America. Several differences defined the two, but the split fell mainly along the lines of an emphasis on environmental influences or experience among the former versus an emphasis on internal or innate influences on the shaping of behavior among the latter.

Experimental psychology

At the turn of the century, Thorndike (1898) demonstrated operant conditioning in cats and Pavlov (1906), classical conditioning in dogs. For the

first time, reliable techniques for measuring the inputs and outputs of a behavior (learning) were available, with no need to refer to mysterious internal states (also known as "the little black box"). Furthermore, in both paradigms, it seemed that animals could be conditioned to an arbitrary stimulus. Encouraged by such results, Watson (1913) and Loeb (1918) took the radical position that internal states are to be ignored not just because their subjective nature is too hard to measure, but because there are no internal states. Animals are biological machines, their behaviors dictated by the environment to which they are subjected. Thus, an adequate explanation of behavior requires no more than a quantitative description of the relationship between the environment (stimulus) and the resulting behavior (response).

This mechanistic stance may seem extreme now (although there still exist proponents). However, what logically followed from an emphasis on stimulus-response was that an explanation of behavior required detailed measurements of their relationship. To measure, a behavioral act had to be precisely defined. This led to the development of a battery of behavioral tests, predominantly to measure learning and predominantly in rats, that are still widely used today. (It also followed that as all animals were stimulus-response mechanisms, an understanding of one should be sufficient to understand all. Thus, the best course of action would be to concentrate on one or a few experimental systems for study.) Led by Skinner (1938), experimental psychology brought a rigor of scientific measurement to the study of behavior that did not previously exist.

The behaviorist school held that, given a "reward" and the proper timing of presentation , any response can be conditioned to any stimulus. However, a set of studies by independent groups (Brown and Jenkins, 1968; Revusky and Garcia, 1970; Wilcoxon et al., 1971) tore that premise apart. Wilcoxon et al. (1971) reported that while rats easily learned to associate a novel taste with illness, they were unable to learn aversion to visual stimuli. Conversely, quail could train to visual but not gustatory stimuli. These results can be easily explained in the context in which these animals normally feed. Nocturnal rats must rely heavily on smell and taste, whereas quail are much more visually oriented animals. In its attempt to quantify behavior, experimental psychology lost sight of behavior's biological relevance. Early on, Jennings (1906) had recognized that animals are not simple stimulus/response machines. The nature of an effective stimulus and subsequent response are subject to heritable constraints, or instincts.

Ethology

Meanwhile in Europe, the ethologists, headed by Lorenz and Tinbergen stressed a more "naturalistic" study of behavior. That is, an attempt was made to relate observed behaviors to the environment to which the species was adapted. Its behavior was then compared to closely related species or species in a similar environmental niche. Behavioral acts shared by several species might be considered to be identifiable "units" of behavior. What was learned from these units might then be generalizable to other animals. In contrast, behaviors unique to a species would likely be less informative. Taking the cue from Darwin, ethology tries to treat behavior as a taxonomic

marker, stressing its evolutionary significance. From this comparative emphasis, it followed that, in contrast to the behavioral psychologists, information on a diverse group of animals should be gathered.

Through studies of this kind, Tinbergen (1948) and others confirmed Jennings' original observations: out of all the stimuli impinging upon an animal at any given time, only a small subset are sufficient to elicit a response. By changing one variable at a time, Marler (1955) demonstrated that the pink breast color in male chaffinches was sufficient to cause them to be treated as males. By removing all other stimuli, with the use of a crude "model," Tinbergen identified the red color of the three-spined stickleback belly as being sufficient to elicit aggressive behavior from other males (1948). In many similar experiments, the key stimulus needed to release the behavior, or the "sign stimulus," for several behaviors were identified.

Once a sign stimulus was recognized, the resulting behavior was often stereotyped and invariant. Even when the stimulus was removed in the middle of the response, the sequence often continued to completion. Moreover, the behavior appeared to be innate and often species-specific. Based upon their observations, Lorenz proposed two general "units" of behavior (Lorenz and Tinbergen, 1938): those which are modifiable by external stimuli, called "reflex patterns," and those that are innate and invariant, termed "fixed action patterns."

Of the two, ethologists seemed particularly enamored with the latter. Their existence provided readily identifiable units of behavior which break up the otherwise steady stream of interactions. Thus, much like operant and classical conditioning for the experimental psychologists, they provided a basis on which to analyze behavior.

Based on these observations, Lorenz (1965) proposed that the control of the form of fixed action patterns was entirely internally mediated by the nervous system. Lorenz predicted the existence of what we today call central pattern generators. It turns out that fixed action patterns are rarely as invariable and resistant to environmental influences as they were first recognized to be. However, their conception has proven a valuable tool to the partitioning, measurement, and understanding of behavior.

With the infallible wisdom that comes only with hind-sight, it seems silly now to focus only on learning or instinct as the primary driving force. For those interested in the study of behavior, the two fields of experimental psychology and ethology complement each other. The former brought the rigor of scientific measurement while the latter put behavior in context of the animal's environment and evolutionary history.

Neural basis of behavior

The basic rationale behind cellular neurobiology is the idea that neurons are the basic processing units in the brain and these by their connections and activity patterns mediate behavior. I state the obvious to outline the paradigm involved. As the nervous system is the physical basis of behavior, one first identifies the players involved by defining the anatomy. This entails identification of neuronal structures by shared features of the neurons within these structures and identification of the gross physical connections between structures and more detailed connections between neurons.

Knowledge of the anatomy allows one to make testable predictions regarding the roles of the structures involved. Based upon these predictions, one can lesion one structure versus another and look at the resulting effect on the behavior of the animal to localize the site of action to one or a few neuronal structures. Knowledge of the anatomy is important. Without it, one cannot draw firm conclusions regarding results from lesion experiments. A failure to see a behavioral defect may be due to residual function from remaining neurons of the same population. A classic example of this kind of pitfall is Lashley's experiments with cortical lesions in the rat (Lashley, 1929). Alternatively, an observed behavioral defect may be the result of indirect damage to another population of neurons.

After the participating neuronal structures have been identified, electrophysiology is used to define functional connections between populations. Knowledge of the anatomy, while essential, is not sufficient, as functional connections do not always correspond to anatomical connections (see below). Additionally, pharmacological agents such as neurotransmitter agonists/antagonists and ion channel blockers can be used to define the physiological properties of these cells.

Researchers have used these techniques to study a variety of vertebrate systems. Since researchers needed to do physiology, historically studies have been done on immobilized animals and thus more is known about the sensory than motor (and other) systems, due to its greater ease. Sensory systems have been studied in owl audition (Konishi et al., 1988), monkey vision (DeYoe and Van Essen, 1988), bat echolocation, and electric fish (Bullock and Heiligenberg, 1986).

However, lesion studies in vertebrates are difficult due to the sheer number and complexity of the system. Lots of redundancy exists in vertebrate cortex and it is hard to demonstrate that a cell or set of cells is necessary for a behavior. For that reason, many researchers turned to invertebrates for their study.

invertebrate cellular neurobiology

The invertebrate organisms offer several features that make them desirable as model systems. Their behaviors are typically more stereotyped than those of vertebrates, making the identification of behavioral "units" easier. Their nervous systems are smaller, making identification and analysis that much simpler. However, is the study of an invertebrate system a valid model for the understanding of higher vertebrates like ourselves? The argument was made that both vertebrates and invertebrates must solve the same problems in the environment (for example, feeding, defense, and mate selection). Thus, at least some of the mechanisms which underlie these behaviors must be similar. The differences will likely come not from the types of mechanisms used but in the complexities and greater capacities of vertebrate systems. Therefore, the issue of "validity" depends on the "level" of analysis at hand.

In the late 60's and early 70's, the existence of large, reproducibly identifiable neurons was discovered (or rediscovered) in several invertebrate systems. These organisms include *Aplysia* (Coggeshall et al., 1967), leech (Nicholls and Baylor, 1968), lobster (Otsuka et al., 1967), locust (Burrows and Hoyle, 1973), and *Tritonia* (Willows, 1968). The identification of these cells

allowed assignment of behavioral function to individual neurons. Armed with this ability, researchers quickly identified central pattern generators (CPGs), functional collections of neurons that produce a rhythmic firing pattern, underling rhythmic behaviors. The defining feature about CPGs is that the rhythms are generated internally, independent of sensory input, as predicted by Lorenz (1965). CPGs have also been found in vertebrate motor systems (reviewed in Grillner and Wallen, 1985).

The rapid progress of these studies made researchers hopeful that an understanding of the basis of behavior was at hand. The widely held belief at the time was that an elucidation of all the connections between neurons would result in an understanding of the behavior. This optimism was one of the reasons that encouraged Brenner to begin study in the nematode, *Caenorhabditis elegans* (see below). This in and of itself is no small task. Reconstruction of the nematode nervous system, which has only 300 neurons and an estimated 5000 chemical synapses took almost ten years to complete (White et al., 1986).

However, several observations made it clear that a cellular understanding would not be as simple as the identification of the parts involved. The parts involved change with time. Known circuits can generate more than one pattern (Getting, 1989; Harris-Warrick and Marder, 1991). Also, one neuron can participate in more than one circuit (Hooper and Moulins, 1989). Not surprisingly, modulation of pattern generators has also been found in vertebrates (Sivgardt, 1989). In addition to changes over minutes and hours, Nottebohm (reviewed in 1989) showed that massive seasonal reorganization occurred in the adult song bird brain system that

mediates singing. This was the first demonstration of "developmental" plasticity in the adult vertebrate.

Owing to this added complexity, the study of invertebrate systems has not delivered on a full understanding of behavior, as was "promised," and their study has since declined in popularity. However, it is clear that invertebrates and vertebrates share many commonalties. Information on motor acts (CPGs) and learning have their correlates in both and in many cases, the understanding at the cellular and molecular levels came from invertebrates first. Even the developmental plasticity and critical periods that are so well studied in vertebrates (Hubel and Wiesel, 1970; Marler, 1987) are now being addressed in invertebrate systems (Hirsch and Tompkins, 1994). Thus, while true that vertebrate systems allow the study of higher order functioning more like our own, the study of invertebrates does allow the elucidation of the same basic mechanisms, and in a system that also allows cell specific knowledge.

In my studies, I have taken advantage of the simplicity of the Caenorhabditis nervous system to perform lesion studies to elucidate the neuronal basis of mating behavior. However, C. elegans does not yet permit electrophysiology of the type done to make the advances discussed in the previous section. As stated, knowledge of the anatomy of the circuits involved does not in and of itself lead to an understanding of how those circuits mediate the behavior. In the absence of physiology, we turn to the next paradigm for behavioral study.

Genetic basis of behavior

Though people may argue over the extent to which heredity influences behavior, few would argue that it doesn't at all. For centuries, man has taken advantage of the fact that behavioral traits are inherited. We select for strains of Betta fish (Siamese fighting fish), cocks, and dogs which are more aggressive. In other situations, we select for animals which tend to be more docile.

In the late 1950's/early 60's, several groups (Hirsch, 1958; Manning, 1961) played with inbred lines of *Drosophila*. Starting with heterogeneous populations, Manning showed that mating speed can be selected for over time. Manning bred for 25 generations, selecting for slow and fast mating speeds. Differences in time were noticeable after only seven generations. By the end, the times varied from 3 to 80 minutes. The studies demonstrated that heritable factors can influence behavior and, most strikingly, showed how fast (i.e. how strong) that influence can be.

Thus, when Seymour Benzer suggested Drosophila as his organism of choice for neurogenetics, he was entering a relatively established system. [The fruit fly had already been very successful as a system for studying development (reviewed in Lewis, 1963).] However, the power of Benzer's approach was that he made it an explicit aim to introduce single-gene mutations into the animal in order to dissect mechanisms of behavior (1973). The paradigm was this: 1) identify a genetic component of behavior (i.e. one that is mutable); identify the genes involved (i.e. screen for mutants); 3) determine the site of action for the identified genes; and 4) determine how they work.

The crucial step is the determination of the site of action. A behavioral defect resulting from the elimination of the function of a gene could result from a number of locations. To address this, Hotta and Benzer (1976) worked out the technique of mosaic analysis in Drosophila. A composite animal is formed, that is wild-type for the gene in question in some tissues and mutant for that gene in others. The mutation is linked to a gene that can be easily (or more easily scored). From the phenotype of the linked gene, one can determine the genotype of tissues in question. By comparing the behavioral defects of mosaic animals that are mutant in some sites versus others, one can deduce whether a wild-type copy of the gene in question is necessary in that tissue. In mouse, an analogous method with chimeras is used (reviewed in Gehring, 1978). For example, Hotta and Benzer (1976), used this technique to determine what parts of the male brain in Drosophila are necessary to mediate male courtship behavior.

Another variable that must be controlled for is time. A resulting behavioral defect from a loss-of-function mutation may indicate a role for the gene in the behavior (i.e.- in the neurons that mediate the behavior). However, the behavioral defect may also be due to developmental abnormalities that only indirectly affect the behavior. This is an issue with the recent gene "knock-out" mutants in mouse (Grant et al., 1992). A resulting defect in spatial learning may be due to loss of an essential ingredient in LTP, in this case CAM kinase II, or due to earlier defects in the development of the granule cells, which also result from this knock-out.

At the time when Benzer started these studies, it was thought that invertebrate behaviors were completely stereotyped so it would be easy to see how they might be genetically coded. This has turned out not to be the case,

but the number of mutants isolated testifies that the idea was still a very good one. The use of genetic screens in Drosophila has led to the identification of many genes involved in various behaviors of the fly, such as learning (Dudai, 1985), courtship (Hall, 1994), olfaction (Siddiqi, 1987), periodicity (Dunlap, 1993). However, the mere identification of genes is not informative. What has been especially powerful is the corroboration of information gained between systems. In work done independently on learning mutants in Drosophila (Quinn, 1974) and Aplysia (Carew and Kandel, 1972) specific molecules were identified that were necessary for learning and memory in both of these systems. Thus, it was highly possible that these molecules were used throughout the animal kingdom.

Another area where invertebrate mutants have proved informative is in olfaction. Buck and Axel have identified the olfactory receptor genes in mouse (1991), moving the study of olfaction to the molecular level. Genes necessary for olfaction have been identified in Drosophila (Siddiqi, 1987) and in nematodes (Bargmann et al., 1993). In these invertebrate systems, the neurons that mediate olfaction are much smaller in number (six in nematodes) and thus easier to analyze. Yet, they work on similar principles of overlapping functions, as do vertebrates (Bargmann and Horvitz, 1991).

The power of a classical genetic screen is that it allows the identification of genes not yet known to exist. Thus, it can identify structures involved in the behavior that cannot be identified with lesion analysis and only with difficulty using electrophysiology. Through use of mutants with known defects in the nervous system, one can emulate pharmacological experiments done in classical electrophysiology with more ease and, in some cases, perform these experiments where it is not possible in physiology.

C. elegans as a system for studying behavior

In selecting *Caenorhabditis elegans* as the system of study, Brenner deliberately chose, not just an animal that was amenable to genetics but one that had a small, reproducibly identifiable nervous system (Brenner, 1973). The latter was especially important because he envisioned a complete reconstruction of the animal's nervous system, such that all neurons and all connections between neurons were identified and accounted for. His belief, which at the time was widely held, was that given this knowledge, one could guess as to how the nervous system worked. Having made those educated guesses, one could go in with genetic analysis, of the kind proposed by Benzer on *Drosophila*, to identify genes which specify development and function of these relatively few neurons.

Characterization of the nervous system: simpler but similar

Through detailed electron microscopy, the nervous system has been completely reconstructed in the hermaphrodite (Ward et al. 1975; Ware et al. 1975; White et al. 1976, 1986; and Hall and Russell, 1991) and partially in the male (Hall and Russell, 1991). Of the 302 neurons in the *C. elegans* hermaphrodite (and 56 additional glial and support cells) most are located in the circumpharyngeal nerve ring in the anterior of the animal or the ventral nerve cord. There is relatively little branching of neuronal processes, and the 302 hermaphrodite neurons make a total of roughly 5000 chemical synapses, 2000 neuromuscular junctions and 600 gap junctions. The male

possesses 79 additional neurons, most of which are located in the copulatory structures of the tail.

While much simpler than vertebrates and even arthropods, the *C. elegans* nervous system, nonetheless, has been shown by immunological and biochemical assays to possess the classical neurotransmitters: acetylcholine (Rand and Russell, 1984); GABA (Schaeffer and Bergstrom, 1988; McIntire et al., 1993a); biogenic amines (Sulston et al., 1975; Horvitz et al., 1982); and neuropeptides (Schinkmann and Li, 1992). Several of the genes encoding these enzymes have been identified. Most recently, McIntire et al. (1993b) demonstrated that some of Brenner's very first behavioral mutants, isolated as Uncs (for uncoordinated), turn out to be defective in the GABA synthesis and release pathway. Another Unc, *unc-6* turns out to be related to laminin, which has been known to be involved in axonal guidance in vertebrates for years (Hedgecock et al, 1989). Thus, while simple, the nematode nervous system is similar in many respects to other systems classically studied for behavior.

lineage

Taking advantage of C. elegans' transparency under Nomarski DIC optics, the entire cell lineage was determined for both hermaphrodite and male, and shown to be essentially invariant (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston et al., 1980, 1983). Knowledge of the cell lineage greatly facilitated two other techniques which have been critical to the ability to study neurobiology in the system without physiology, cell lesion studies via photoablation (Sulston and White, 1980) and mosaic analysis

(Herman, 1984). The usefulness of these two techniques has already been discussed in previous sections. Cell lesions or ablations are performed by irradiating a targeted cell nucleus with a pulsed dye laser. The small number of neurons in this animal and a complete knowledge of their lineal histories allows the reproducible targeting of single neurons.

genetics

C. elegans has many features that make it attractive as a model system for the study of behavior. It exists primarily as a self-fertilizing hermaphrodite, with a short generation time, that allows facile genetic and developmental studies. The given estimated genome size is less than 5,000 genes. (However, more recent estimates via molecular analysis have increased that number.) To date, between 250-300 genes have been reported which specifically affect the nervous system. Starting with Brenner's first screen for uncoordinated mutants, genes have been identified for many behaviors: pharyngeal pumping (Avery, 1993); chemosensation and olfaction (Bargmann, 1991 and 1993); touch response (Kaplan, 1994); turning (Loer, 1993); and defecation (Thomas, 1990). In addition, genes have been found which are involved in general function of the nervous system, (see above).

Male mating behavior in C. elegans

I chose to study mating behavior in C. elegans because of the convenient combination of a complex behavior in a relatively simple nervous

system. From the analysis, I hoped to learn more about the initiation and coordination of behavioral acts or steps in a complex behavior. To do these analyses, I have used a combination of all three approaches discussed above. As the following chapters will describe, I started off my investigations with a quantitative description of the behavior. This allowed me to better recognize possible changes in behavior following lesion. Then, I performed lesion or ablation analyses to identify the cells involved in the behavior. Assuming that male specific neurons mediate a male specific behavior, I targeted the cells generated by post-embryonic lineages in the male. This turned out to be not a bad assumption since most of these cells are associated with the copulatory structures. Results of these analyses went into a paper which is chapter 2. Some additional cellular analysis is appended in that chapter. The results showed that male mating behavior is not completely hard wired but rather controlled by sensory feedback at each step.

Simultaneously with the ablations, I performed a genetic screen for mutants defective specifically in copulation, called Cods for copulation defective. Our first screen failed to pick up defects in vulva location. The results were understandable, given the ablation results in chapter 2. However, a revised screen, based on our findings, successfully isolated mutations in each step of the behavior, suggesting independently of the ablation studies that each step is independently regulated. In addition, I also screened previously isolated mutants for defects in mating behavior, the results of which are described in the appendix to chapter 3.

Both the ablation and genetic results suggested feedback. Obviously then, the hermaphrodite must be producing needed signals. Analysis of the

hermaphrodite generated signals is in chapter 4. Finally, evidence of learning during mating is in the appendix at the end of the thesis.

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Chapter 2

Sensory Regulation of Male Mating Behavior in *Caenorhabditis elegans*

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Sensory Regulation of Male Mating Behavior in Caenorhabditis elegans

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Summary

C. elegans male mating behavior comprises a series of steps: response to contact with the hermaphrodite, backing along her body, turning around her head or tail, location of the vulva, insertion of the two copulatory spicules into the vulva, and sperm transfer. By ablation of male-specific copulatory structures and their associated neurons, we have identified sensory structures and neurons that participate in each of these steps: the sensory rays mediate response to contact and turning; the hook, the postcloacal sensilla, and the spicules mediate vulva location; the spicules also mediate spicule insertion and regulate sperm transfer. Generally, successful completion of each step places the male in a position to receive a cue for the next step in the pathway. However, the high degree of sensory regulation allows the male to execute some steps independently.

Introduction

Understanding the cellular basis of a behavior requires that the neurons mediating sensory input, any internal processing, and the motor output all be identified and delineated in a pathway by both morphology and physiology. This daunting task can be simplified by studying invertebrate systems with their smaller nervous systems and often stereotyped behaviors, which allow better control of measurements and hypothesis testing. The task can be simplified further by focusing on the question of how a response behavior is generated and modified. Two experimental approaches to answer this question are the identification of the parts of the nervous system that mediate this behavior (by means of lesions and electrophysiology) and the elucidation of the mechanisms by which these neurons serve their function (by means of electrophysiology and genetic and molecular analysis). The large, identifiable neurons of such organisms as lobster, leech, and Aplysia have allowed the functional mapping of neuronal circuits. In contrast, whereas electrophysiology is limited in Drosophila and nearly nonexistent in Caenorhabditis elegans (however, see Raizen and Avery, 1994), these two systems are amenable to genetic analysis. For example, Drosophila neurogenetics has shed light on learning, biological clocks, and courtship behavior (Quinn and Greenspan, 1984; Davis, 1993; Dunlap, 1993; Hall, 1994).

In addition to genetic analysis, cell ablations in C. elegans can be used to assign behavioral roles to neurons (developed by Sulston and White, 1980; modified by Avery and Horvitz, 1987, 1989). Candidate neurons are ablated with a laser microbeam, and the behavior of the operated animal is compared with the behavior of intact animals. Behavioral defects, if any, suggest that the ablated neurons are involved in that behavior. As the number of neurons in the animal is small (302 in the hermaphrodite, 381 in the male) and as each neuron is identifiable by lineage and position, systematic ablation of individual cells is feasible. The combination of ablation of identified cell types and genetic studies has led to dissection of a significant part of the worm behavioral repertoire, including response to touch (Chalfie et al., 1985; Kaplan and Horvitz, 1993), chemotaxis (Bargmann and Horvitz, 1991), and pharyngeal pumping (Avery and Horvitz, 1989).

Compared with the above behaviors, male mating behavior, comprising a series of sub-behaviors or steps, is arguably the most complex behavior exhibited by this small nematode. Yet each step in the behavior is highly stereotyped, suggesting an innate motor program. This reproducibility allows description, reported by Hodgkin (1974), Dusenbery (1980), Baird et al. (1992), and J. Sulston (personal communication), and extended by us. When the posterior part of a C. elegans male comes into contact with a hermaphrodite, the male responds by placing the ventral side of his tail against her and proceeding to swim backwards along the length of her body (Figure 1A), turning via a deep ventral flexion around either her head or tail (Figure 1B), until he locates the hermaphrodite vulva. At this point, he stops (Figure 1C), inserts a pair of copulatory structures called the spicules (Figure 1D), and transfers sperm. Hermaphrodites, which are internally self-fertilizing, appear to play little or no active role in this process.

In organisms as distantly related as rats (Breedlove, 1986) and insects (Schneiderman and Hildebrand, 1985), sex-specific behaviors result from sexually dimorphic nervous systems. In C. elegans, one of two classes of hermaphrodite-specific neurons mediates the hermaphrodite-specific behavior of egg laying. Male-specific mating behavior is likely mediated by the male-specific neurons. As a testament to the complexity of the behavior, in contrast with the hermaphrodite, which has only 8 sex-specific neurons, the male has 87 (almost a quarter of the entire male nervous system). All but 4 of these neurons are associated with a set of male-specific structures collected in the posterior part of his body, referred to as the male tail (Figure 2).

The structures of the male tail have been described by Sulston et al. (1980). From anatomical studies, they are all believed to be sensory. Each male is endowed with 9 bilateral pairs of sensory rays that spread out on both sides of the male (Figure 2A); each ray is composed of 2 neurons and a structural cell. On the ventral side, just anterior to the cloaca, is a sensory organ called the "hook," which consists of 2 sensory neurons, 2 support cells, and a structural cell. Just posterior to the cloaca are a pair of bilaterally symmetrical sensilla, called the postcloacal sensilla. Each sensilla contains 3 sensory neurons and 3 support cells. Within the cloaca, there exists a pair of cuticularized structures called the spicules (Figure 2B); each spicule is com-

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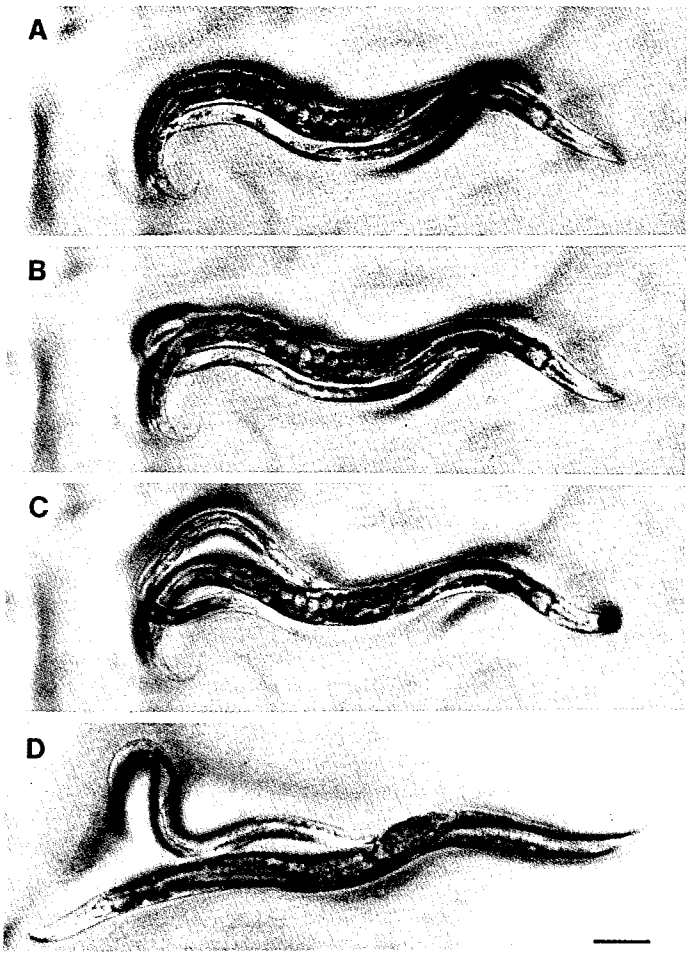

Figure 1. The Sequence of Steps in Male Mating Behavior in C. elegans

(A) Response to contact with the hermaphrodite. The male arches the posterior third of his body such that the ventral side of his tail, containing the copulatory apparati, is apposed to the hermaphrodite.

(B) Male turning from the dorsal side of the hermaphrodite tail to the ventral side. The turn, consisting of a deep ventral flexion of the male's tail, is initiated before he reaches the end of the hermaphrodite.

(C) Male at the hermaphrodite vulva.

(D) Insertion of the copulatory structures called the spicules. Bar, 0.1 mm.

posed of 6 structural cells, 2 sensory neurons, and a motor neuron also thought to be proprioceptive in nature.

In our analysis, we have quantified many of the steps of mating behavior to better understand the behavior in the intact animal and to serve as a basis for determining defective behavior in altered animals. Subsequently, we ablated male-specific structures and associated neurons to infer their roles in mating behavior.

Results

Mating Behavior in Intact Animals

Here we discuss mating behavior in the intact animal and our criteria for behavioral defects. For all measurements given, n = 23, unless otherwise stated.

Response to Contact

When the male tail comes into contact with a hermaphrodite (Figure 1A), the male responds by apposing the ventral side of his tail to her body and swimming backwards along the length of her body. If contact is made with the ventral side of the male tail, he proceeds to swim backwards, keeping the posterior third of his body rigid against her (the rest of the body moves in a sinusoidal motion that is normal for swimming). If contact is made with the dorsal side, response entails swimming backwards with a ventral arching of the posterior sixth of his body until the ventral side comes into contact with the hermaphrodite. Thus, what we call response to contact is composed of three parts: the halting of forward motion, the placement of the ventral side of the male tail against the hermaphrodite, and the start of backwards swimming. To be classified as defective in response to contact, the males must fail to perform the last two substeps.

Turning

As the male approaches either the hermaphrodite head or tail (Figure 1B), he turns around the head or tail to the other side of the hermaphrodite via a sharp ventral arch of his tail. The turn is initiated before he reaches the end of the hermaphrodite, with approximately one-twelfth (estimated by eye from observation and micrographs) of the body length left to travel. Thus, there are two components to a proper turn: a sharp ventral arch (executed by male-

specific muscles and motoneurons [Loer and Kenyon, 1993]) and proper timing of the arching behavior. Even if he completes a turn, a male is considered defective in this step if either one of these components is defective.

Vulva Location

The male continues to swim backwards until he locates the hermaphrodite vulva, where he stops (Figure 1C). More than 95% of the time, males stop backing upon first encounter with the vulva (taking about 8.5 ± 1.5 s). Occasionally, the male will stop beyond the vulva. If he is within 10% of a hermaphrodite body length, he will more likely back up slowly (i.e., swim forwards) to relocate the vulva. Beyond that distance, he will more likely continue to swim backwards until he encounters the vulva again. Once the male locates the vulva, he slows his swimming speed and adjusts his position via a short back and forth motion, covering about 10% of the hermaphrodite body. During this time, the male's spicules are extruded, the posterior third of his body is no longer kept rigid, and his pharyngeal pumping rate decreases dramatically from an average of 180 pumps per minute to 50 ± 16 (n = 16).

Thus, what we call vulva location is composed of two parts: the cessation of backward motion along the hermaphrodite, at the approximate location, and the adjustment of position via a slow back and forth motion in the vicinity of the vulva, to find the precise location. For the purposes of scoring behavioral defects, brief hesitations around the vulva without subsequent adjustment behavior were not considered sufficient for vulva location behavior.

Spicule Insertion

Spicule insertion and adjustment of vulva location are functionally equivalent and behaviorally inseparable except by ablation (Figure 1D). The spicules are extruded while the male searches for the precise location of the vulva, and they can be seen depressing the hermaphrodite cuticle. The male continues to extrude spicules until they are inserted into the vulva, anchoring the male in place.

For reasons that are unclear, spicule insertion is a difficult step for the males to accomplish. In almost three-quarters of the attempts, males swim away after being unable to insert their spicules, after attempting for an average of 210 ± 45 s. Observations indicate that this spicule insertion "problem" is hermaphrodite specific (see Experimental Procedures). If a male does succeed in inserting his spicules, it usually happens quickly, taking an average of 20 ± 2 s.

Sperm Transfer

After spicule insertion, sperm is released from the seminal vesicle, through the vas deferens and cloaca and into the vulva (between 30–180 sperm per mating). The anal sphincter is contracted at this time, presumably to open the cloaca, but the active mechanism, if any, that transports the sperm is not known (Sulston et al., 1980). The time it takes for sperm transfer is about 4 s (from the start of fluid flow to the end). The male continues to keep his spicules inserted after the completion of sperm transfer for about 27 ± 5 s. In other Caenorhabditis species and in the *plg-1* strain, males lay down a copulatory plug over the vulva at this time (J. Hodgkin, personal communications; unpublished data; Barker, 1994). Presumably, the "plugless" laboratory (Bristol) strain has retained the quiescent behavior. After the male "releases" the hermaphrodite, the spicules slowly retract, taking about another 20 ± 3 s. The male slowly begins swimming forwards again and foraging, with pharyngeal pumping returning to normal.

Cell Ablations

Rationale

To determine the possible roles of male-specific sensory structures in male mating, we ablated them individually and in combinations and observed subsequent mating behavior for defects. Our strategy was first to ablate blast cells from which the sensilla are derived, eliminating entire groups of male-specific cells. If a behavioral defect was observed, the progeny of the blast cell were then systematically ablated, following the behavioral defect to the single-cell level. Daughter cells that did not give rise to the behavioral defect serve as internal controls for sisters that did, as these animals were subjected to the same treatment as those that did exhibit the defect. If at any time the defect was not attributable to a single cell, both daughter cells were ablated to determine whether the behavior is mediated by both cells or their progeny. If ablation of a blast cell did not give rise to a behavioral defect, other cells that are candidates for a redundant function were ablated in conjunction. Because of the large number of cells in question, not all combinations could be tested, but many logical combinations (because of proximity, type, lineage, or known function) were. In this way we were able to identify systematically the roles of many of the male-specific neurons in male mating behavior.

This approach addresses another potential problem: when we eliminate a precursor cell, we can be certain that its progeny and their function have been removed. However, it is possible that the targeted cells may be replaced by a neighboring, intact cell. Thus, lack of an observed defect may be due to replacement of a necessary cell. Conversely, a noted defect may be due to loss of a neighboring cell to replace the ablated cell. Ablation of terminal cells in the lineage avoids this problem of possible regulation of cell fates. However, it is conceivable that cell function is not completely eliminated (but see Avery and Horvitz, 1989). We therefore used both protocols when possible.

For the purposes of these studies, we assume these neurons are correctly classified as sensory. Functional studies of hermaphrodite neurons, also classified as sensory by morphology, have verified their classification (Davis et al., 1986).

The Sensory Rays

The blast cells V5L/R.p, V6L/R.p, and TL/R.ap generate 9 bilateral pairs of sensory rays, numbered 1 (anterior) to 9 (posterior) (Figure 2A). Each ray is composed of a single structural cell and 2 sensory neurons, with processes that extend the length of the ray. The sensory neurons are designated RnA and RnB, where n is the number assigned the ray. The neurons have access to the outside via a sensory opening in all ray pairs but ray 6 (Sulston and Horvitz, 1977; see also Baird et al., 1991). Because of the large number of rays, we divided them into subgroups

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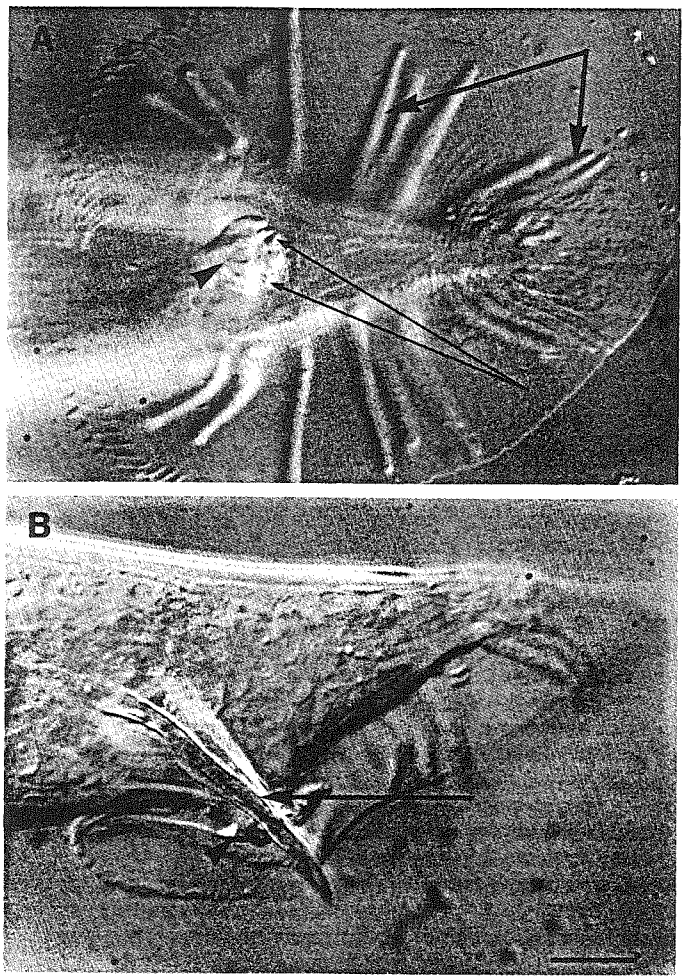


Figure 2. The Male Tail Contains All of the Copulatory Structures of the Male and Most of His Sex-Specific Neurons

(A) Nomarski micrograph of a ventral view of the male tail showing 9 bilateral pairs of sensory rays (thick arrows), the hook and associated sensillum just anterior to the cloaca (arrowhead), and a pair of postcloacal sensilla (thin arrows), named because of their position.

(B) Lateral view of the male tail (ventral is down) showing the left homolog of a pair of extendible cuticular structures called the spicules (thick arrow). The hook (arrowhead) and some rays can be seen as well. Bar, 0.01 mm.

based on location of their sensory openings (Table 1), position of rays along the animal (Table 2), and neurotransmitter phenotype.

The sensory openings of rays 1, 5, and 7 open to the dorsal side of the animal. The sensory openings of rays 2, 4, and 8 open ventrally. Rays 3 and 9 open laterally. Ray 6 has no sensory openings to the external environment. To determine the significance of the positions of these sensory openings, we ablated only the dorsally opening rays. Operated males fail to respond to dorsal contact with the hermaphrodite but respond normally to ventral contact. We infer that the rays require direct contact with the sensory openings to mediate response to the hermaphrodite. Ablation of the ventrally opening rays results in no observable defect. Ablation of the laterally opening rays and ray 6 also leads to no observable defects, either alone or in combination with any rays other than the dorsally opening rays. Ablation of all but the dorsally opening rays gives no observable defect. Thus, the dorsally opening rays are both necessary and sufficient to mediate response to dorsal contact. Also, the lack of a defect after ablation of the ventral rays is not due to redundancy of function with the lateral or closed rays.

To test whether the ability to respond to contact after the ablation of the ventrally opening rays is due to the presence of additional ventral sensory structures, namely the hook, postcloacal sensilla, and spicules, we ablated all ventral organs via their precursors and the ventrally opening rays (see above). These animals do not respond to ventral contact with the hermaphrodite. In response to dorsal touch, the males curl their tails ventrally, reminiscent of contact response behavior, but do not pursue contact (backing) with the hermaphrodite. When only the hook, postcloacal sensilla, and spicules are ablated, leaving the ventrally opening rays intact, the operated males respond to ventral (and dorsal) contact and back along the hermaphrodite, but fail to locate the vulva. This observation suggests that the ventral sensory structures are redundant with the ventrally opening rays for the maintenance of search behavior along the hermaphrodite. However, the presence

Table 1. Sensory Ray Ablations by Position of Sensory Opening

Structures Ablated	Effect (removes)	Response to Dorsal Contact		Response to Ventral Contact	
		By Males	By Trial	By Males	By Trial
None	None	10/10	97/100	10/10	98/100
Rays 1, 5, 7	All dorsal pairs	0/10	0/80	10/10	24/24
Rays 2, 4, 8	All ventral pairs	10/10	64/66	10/10	45/45
Rays 3, 9	Both lateral pairs	10/10	52/52	10/10	24/24
Ray 6	Closed pair	10/10	44/44	10/10	21/21
Rays 2, 3, 4, 6, 8, 9	All but dorsal pairs	7/7	46/48	7/7	35/36
Rays 2, 4, 8 plus hook, p.c.s., spicules	All ventral sensilla	8/8[a]	64/64	0/8	0/22
Hook, p.c.s., spicules	All non-ray ventral sensilla	10/10	99/100	10/10	96/100

p.c.s., postcloacal sensilla.
[a] All males exhibited ventral arching of the tail upon dorsal contact with hermaphrodites but did not pursue hermaphrodites following either dorsal or ventral contact.

of the ventral sensory structures alone is not sufficient to mediate the initiation of backing behavior. Hence, the initiation of backing behavior and its maintenance are mediated by distinct sets of sensilla.

Because the male swims backwards during mating behavior, the most posterior rays precede the rest of the animal. The 3 most posterior pairs of rays are generated by the bilateral pair of blast cells, TL/R.ap (which also generate two hypodermal cells and a non-sex-specific interneuron). The 6 more anterior pairs of rays are generated from the blast cells V5L/R.p and V6L/R.p (which also generate a pair of sensilla called the postdeirids and some hypodermal cells).

Ablation of V5L/R.p and V6L/R.p, which generate the 6 anterior pairs of sensory rays, results in males missing these rays. These males are incapable of responding to contact with hermaphrodites, in that they continue to swim forwards, passing the hermaphrodite. This lack of response is not simply due to insufficient numbers of rays remaining to transmit a signal (the remaining 3 pairs of T-derived rays). Ablation of the T-derived rays along with 3 of the 6 V-derived rays results in males that respond at normal efficiency. This control demonstrates that 3 pairs of rays are adequate for mediating response to contact; however, it does not account for position. It may be that the T-derived rays are not in the position to serve this function, being at the most posterior end of the male. There is no obvious difference in morphology between the T- and V-derived rays; thus, the functional difference between them may result from differences in position and wiring rather than innate differences in the two lineages.

Ablation of TL/R.ap, which gives rise to the 3 most posterior pairs of rays, results in males that are missing the T-derived rays. Whereas intact males initiate a turn (deep ventral flexion of the posterior quarter of the male) at the last twelfth of the hermaphrodite body (Figure 1B), TL/R.ap-ablated males swim past the end of the hermaphrodite, losing contact and ending with their tail in a tight ventral coil (Figure 3B). These observations suggest that the males fail to turn at the appropriate time, while the tight ventral coil remains intact.

The A neurons of rays 5, 7, and 9 express dopamine and are likely dopaminergic (Sulston et al., 1975). Ablation of these neurons results in males that turn at the end of the hermaphrodite but tend to do so in a large, sloppy arc instead of the tight ventral coil seen in intact animals (Figure 3C). These observations suggest that the males have kept the timing of the turn intact but have lost the

Table 2. Sensory Ray Ablations by Position of Rays along Anterior–Posterior Axis

Structures Ablated	Effect (removes)	Response		Turning	
		By Males	By Trial	By Males	By Trial
None	None	10/10	100/100	10/10	100/100
Rays 1–6	6 most anterior pairs (V rays)	0/10	0/100	NA	NA
Rays 7–9	3 most posterior pairs (T rays)	14/14	140/140	0/14	0/140
Rays 1–3, 7–9	All but 3 middle pairs	10/10	58/62	0/10	*
Rays 4–6, 7–9	All but 3 most anterior pairs	10/10	49/49	0/10	*

NA, not applicable.
* Turning defects were not recorded in these experiments, as it had already been demonstrated that ablation of the T-derived rays eliminated turning. These ablations were done to see whether they had any effect on response.

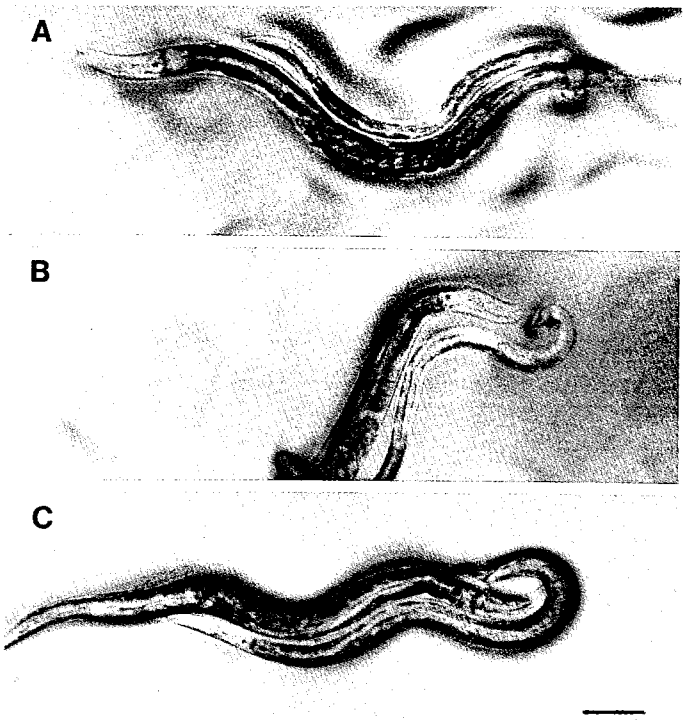


Figure 3. Comparison of Turning Defects Resulting from Sensory Ray Ablations

Note the position of the hermaphrodite head/tail in relation to the male tail.

(A) Turning in the intact male. Males turn via a sharp ventral flexion of the posterior before they reach the end of the hermaphrodite.

(B) Ablation of the posteriorly positioned T-derived rays results in males that fail to turn at the appropriate time. Instead, they swim off the end of the hermaphrodite, coiling ventrally.

(C) Ablation of the dopamine-containing rays (5, 7, and 9) results in males that turn in a wide loop, as opposed to the sharp ventral coil of the intact male. Bar, 0.1 mm.

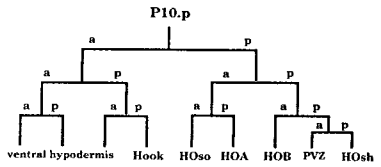
tight ventral coil. Ablation of the corresponding B neurons results in males with no behavioral defects. The RnA ablation defect is similar to the behavioral defect of *cat-2* males (K. Liu, unpublished data), which are dopamine deficient (Sulston et al., 1975).

The Hook Sensillum

The hook sensillum comprises 2 sensory neurons (HOA and HOB) and 2 support cells and is associated with the hook, a single cell–derived, hook-shaped, sclerotic structure. Ablation of P10.p eliminates all of these cells in addition to an interneuron and 3 epidermal cells (Figure 4); morphology is normal, except that the hook and associated sensillum are missing. Operated males fail to stop at the vulva, circling the hermaphrodite many times, yet yield cross-progeny (Sulston and White, 1980; unpublished data). Eventually, the males stop, extrude their spicules, and begin to back along the hermaphrodite at a much slower pace, displacing her cuticle with their spicules as they back, until they locate the vulva by slipping the spicules into the vulva. This alternative form of vulva location behavior is discussed below.

Ablation of P10.pp eliminates both hook-associated sensory neurons, 2 neuronal support cells, and an unrelated interneuron, but leaves the hook structure intact (Figure 4). Operated males pass the vulva as before. Ablation of either hook neuron HOA or HOB alone (P10.ppap or P10.pppa, respectively) results in males that are similarly impaired. These males occasionally hesitate around the area of the vulva but continue swimming and do not try to insert their

A



B

Cell(s) Ablated	Structure Missing	Vulva location by male	Vulva location by trial
none	none	10/10	97/100
P10.p [a]	hook & sensillum +	0/10	0/100
P9.p [b]	none	4/4	13/13
P10.pa	hook +	10/10	33/37
P10.papp	hook alone	11/11	32/35
P10.paa	hypodermal cells	3/3	10/10
P10.pp	sensillum +	0/12	0/120
P10.ppap	HOA	0/10	0/100
P10.pppa	HOB	0/10	0/100
P10.ppap & pppa	both hook neurons	0/7	0/70
P10.ppaa	HOso	6/10	6/62
P10.ppppp	HOsh	3/10	3/88
P10.ppppa	PVZ	10/10	42/44

Figure 4. Role of the Hook and Sensillum

(A) The P10.p lineage from which the hook is derived. The hook is composed of a structural cell and a sensillum, containing 2 neurons and 2 support cells. HOso, hook socket cell; HOsh, hook sheath cell. Lineage from Sulston et al., 1980.

(B) Results of ablations of the P10.p ectoblast and its descendants. The plus sign indicates that additional structure/cells were removed with this ablation.

[a]Since P9.p has been shown to replace P10.p after ablation at this stage (Sulston and White, 1980), both P9.p and P10.p were ablated to remove P10.p functionally.

[b]Analogously, both P8.p and P9.p were ablated.

Table 3. Analysis of Vulva Location Behavior

Cell(s) Ablated	Structure(s) Missing	Vulva Location Behavior		Mating Efficiency
		Approximate	Precise	
Observations with Intact Hermaphrodites				
None	None	Stops at vulva	Slow search using spicules	High
P10.p	Hook	Circles hermaphrodite	Finds vulva via slow search	Low
Y.pl/r	p.c.s. (except PCC)	Stops at vulva	No slow search; loses vulva easily	High
Y.pl/r, B.al/rpaaa	p.c.s.	Stops at vulva	No slow search; loses vulva easily	High
P10.p, Y.pl/r	Hook, p.c.s. (except PCC)	Circles hermaphrodite	No slow search	Very low
P10.p, Y.pl/r, B.al/rpaaa	Hook, p.c.s.	Circles hermaphrodite	No slow search	0
B.al/rpa	SPC, SPD, PCC	Stops at vulva	No slow search	0
B.β	SPV	Stops at vulva	Slow search	Very low
P10.p, B.al/rpapap	Hook, SPD	Circles hermaphrodite	Slow search; no spicules	0
Observations with vulvaless hermaphrodites				
None	None	Circles hermaphrodite	No slow search	NA
P10.p	Hook	Circles hermaphrodite	No slow search	NA

Vulva location behavior is divided into two substeps, here designated "approximate" and "precise," as explained in the description of vulva location behavior in intact animals. Mating Efficiency is explained in Experimental Procedures. For observations with intact hermaphrodites, n = 10; vulvaless hermaphrodites, n = 14 (None) or n = 6 (P10.p). p.c.s., postcloacal sensilla.

spicules at that time. Thus, neither neuron alone is sufficient to mediate vulva location. Ablation of both HOA and HOB is equivalent to ablation of the hook sensillum, indicating that both are necessary for proper hook function. Ablation of the support, socket (P10.ppaa), and sheath (P10.ppppp) cells also impairs vulva location, although to a lesser extent. The socket and sheath cells are thus necessary for proper function or differentiation of the HOA and HOB neurons.

Ablation of P10.pa, which eliminates the cell that forms the hook structure and 3 epidermal cells, results in males that have only a sensillum where the hook is normally located (see also Sulston and White, 1980). These males are capable of locating the vulva, indicating that the structure itself is not necessary for hook sensillum function (Figure 4). This observation supports the idea that the hook functions in a chemosensory manner. However, ablation of the hook structural cell does result in a minor defect, in which the males consistently stop slightly beyond the vulva. Subsequent examination using Nomarski optics shows that the remaining hook sensillum, while intact, is often displaced anteriorly. Therefore, we believe that, though the hook structural cell is not necessary for sensillum function, the position of the sensillum might be important for efficient vulva location.

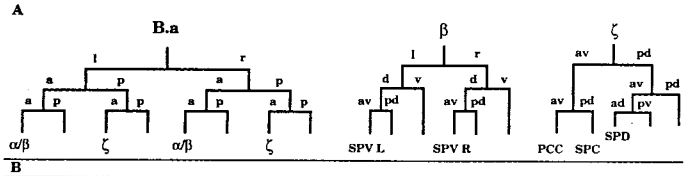
The Postcloacal Sensilla

Each postcloacal sensillum is made up of 3 sensory neurons (PCA, PCB, and PCC) and 3 support cells. The blast cell Y.p generates all of these cells exclusively, with the exception of the PCC neurons, which are generated by the B.a cell. Ablation of the entire postcloacal sensilla (by ablation of Y.pl/r and B.al/rpaaa) results in males with normal morphology and almost normal mating behavior (Table 3). (Since ablation of Y.p in L1 larvae often results in deformed spicules [Chamberlin and Sternberg, 1993], we ablated the progeny Y.pl/r in the L2.) Operated males respond, turn, and readily stop in the general area of the vulva, indicating that they recognize it. However, they tend to lose the vulva while attempting to locate its precise position (normal behavior exhibited in 0/10 males, 6/100 trials). Mating efficiency in these animals is normal (see Experimental Procedures).

Ablation of any 2 of the 3 pairs of postcloacal sensory neurons results in males with a similar but less severe defect (PCA^-PCB^-, 0/8 males, 12/80 trials; PCA^-PCC^-, 0/2 males, 7/20 trials; PCB^-PCC^-, 0/3 males, 9/30 trials). Ablation of any 1 pair results in no observable defect (PCA^-, 6/6 males, 18/19 trials; PCB^-, 7/7 males, 20/24 trials; PCC^-, 6/6 males, 18/20 trials). Therefore, a single pair of postcloacal sensilla sensory neurons is insufficient for the postcloacal sensilla to function; 2 pairs are sufficient but not optimal. Whereas the 3 pairs of sensory neurons seem interchangeable and overlapping in function, their number is important.

Although males in which the hook has been ablated (P10.p) cannot locate the vulva using the usual method, they eventually locate the vulva by extruding their spicules and backing slowly. This slow search behavior is similar to that observed in the vicinity of the vulva in intact animals, during adjustment to find the precise location of the vulva. Ablation of the hook and the postcloacal sensilla together results in males that neither hesitate around the vulva nor adopt the slow search behavior (Table 3). Ablation of the SPD sensory neurons (located in the spicules; see below) along with the hook also impairs vulva location. From this observation, we infer that, whereas the hook is responsible for finding the approximate location of the vulva, the postcloacal sensilla, in conjunction with the spicules, is used to find the precise location. In the absence of cues from the hook, the postcloacal sensilla can be used with the spicules to perform the same function.

A



B

Cell(s) Ablated	Neurons Missing	by male	by trial	Defect
none	none	10/10[a]	30/30[a]	--
B.a	all	0/10	0/100	no spicule insertion
Bζ	SPC, SPD, PCC, +	0/12	0/120	no spicule insertion
Bζaa	PCC	6/6	18/18	--
Bζap	SPC	0/10	0/100	no spicule insertion
Bζpaa	SPD	2/10	2/95	no spicule insertion
Bζpap	SPsh	5/5	15/15	--
Bζpp	SPso	6/6	18/18	--
Bβ	SPV +	1/12	2/112	no sperm transfer (31/112) premature transfer (79/112)
Bβl/rda	SPV	1/10	1/93	no sperm transfer (17/93) premature transfer (75/93)
Bζap, Bζpaa	SPC, SPD	0/4	0/40	no spicule insertion
Bζap, Bβl/rda	SPC, SPV	0/4	0/40	no spicule insertion
Bζpaa, Bβl/rda	SPD, SPV	0/3	0/30	no spicule insertion

Figure 5. Role of the Spicules

(A) The B.a lineage from which the spicules are derived. The spicules each contain 2 sensory neurons and a motor neuron that is thought to act as a proprioceptor as well. (α/β) In a given male, either B.alaa or B.araa becomes α; the other becomes β; (ζ) B.al/rpa. Lineage from Sulston et al., 1980.

(B) Results of ablations of the B.a ectoblast and its descendants. The plus sign indicates that additional structure/cells were removed with this ablation.

[a]Even intact males cannot insert their spicules much of the time. Observations have shown that the reason is hermaphrodite specific (see Experimental Procedures, Observations of Behavior). For these observations, we selected hermaphrodites that allowed spicule insertion by intact males.

The slow search behavior is not seen in intact males paired with hermaphrodites lacking vulvae (either vulvaless *let-23(sy1)* mutants or hermaphrodites where the vulva has been eliminated via ablation; Table 3). Thus, either the slow search behavior is vulva dependent, or it is an aberration resulting from ablation of the hook (and rescued by further ablation of the postcloacal sensilla). Pairing hook-ablated males with vulvaless hermaphrodites also results in no alternative behavior, suggesting that a signal from the vulva must be sensed by the postcloacal sensilla for the male to adopt this alternative behavior. Thus, even though the male cannot find the precise location of the hermaphrodite vulva while circling, he must be responding to some general cue from, or released through, the vulva.

The Spicules

Each spicule contains 2 sensory neurons, SPD and SPV, the processes of which run down the length of the spicule to the sensory opening at the end. Each spicule also contains a motor neuron, SPC, which appears, by morphology, also to be proprioceptive in nature (Sulston et al., 1980). Ablation of the blast cell B.a eliminates all of these neurons plus a pair of sensory neurons from the postcloacal sensilla PCCL/R (Figure 5). In addition, the majority of the structural cells of the spicules are also eliminated. The operated male has no spicules, and as expected, spicule insertion and sperm transfer do not occur. There are no other discernible defects (ablation of PCCL/R has no noticeable effect, as their function is redundant with PCAL/R and PCBL/R; see above).

Ablation of the Bζ cell (nomenclature from Chamberlin and Sternberg, 1993) eliminates only the SPC, SPD, and PCC sensory neurons and 4 support cells (a pair of socket cells and a pair of sheath cells). Despite lacking 2 structural cells each (out of 6), the spicules appear structurally intact. These animals fail to insert their spicules into the vulva (Figure 5). Ablations of the SPC motor neurons (Bζap) or the SPD sensory neurons (Bζpaa) also result in failure to insert spicules. Ablation of the PCCs (Bζaa) has no discernible effect. Neither does ablation of either pair of structural cells (Bζpap/Bζpp), implying that these cells are not necessary for proper neuronal differentiation or function.

Ablation of Bβ eliminates the SPV sensory neurons and 4 spicule support cells (2 socket and 2 sheath) but leaves the spicules intact. With most ablated animals, sperm are occasionally released outside of the vulva (Figure 5), before the spicules have been properly inserted. In all but a few of the remaining trials, the males fail to release sperm altogether once the spicules have been inserted.

Ablation of both the SPC motoneurons and the SPD sensory neurons resulted in males defective in spicule insertion, as expected. Ablation of the SPV sensory neurons, in conjunction with either the SPCs or the SPDs, also resulted in males defective in spicule insertion. No defects in sperm transfer were observed. These results imply that the function of the SPV sensory neurons is dependent upon the function of both the SPD and SPC neurons.

Discussion

We have described in detail the steps in male mating behavior in C. elegans. We then examined the roles of the sensory neurons/structures of the C. elegans male tail, via ablation and observation of the resulting behavioral defects, and assigned most to specific roles in these steps.

Response to Contact

Response to contact with the hermaphrodite is mediated through the sensory rays. For this function, the V-derived rays appear to be more important, as they are capable of mediating contact response in the absence of the T-derived rays; the converse is not true. This functional difference is more likely due to differences in ray position rather than the number of rays. Direct contact of the hermaphrodite with the sensory openings of the rays is necessary, as shown by the dorsally opening rays being both necessary and sufficient to mediate response to contact with the dorsal side of the male. However, response to ventral contact

appears to be mediated through both the ventrally opening rays and the additional sensory organs on the ventral side.

Turning

Once the male is backing along the length of the hermaphrodite body, he might encounter either her vulva or her head or tail first. If he encounters the end of the hermaphrodite before reaching the vulva, he will turn around to her other side. Turning is also mediated through the sensory rays. There are two components to a properly executed turn: timing and ventral coiling. The T-derived rays (rays 7–9) appear to mediate the timing, as operated males coil too late, swimming off the end of the hermaphrodite. The dopamine-containing rays (or the dopaminergic neurons alone), rays 5, 7, and 9, appear to mediate the ventral coil, as these operated males make wide sloppy turns instead of the sharp ventral arches exhibited by intact males.

Vulva Location

Once the male arrives in the vicinity of the vulva, he ceases backing behavior and adjusts his position before spicule insertion. Our observations suggest that this is a two step process involving distinct sensilla. The role of the hook and its associated sensillum is to identify the approximate location of the vulva over the entire area of the hermaphrodite body. Once the male has stopped in the general area of the vulva, the postcloacal sensilla in conjunction with the spicules act locally to determine the precise location of the vulva.

In the absence of the hook, males are still able to locate the vulva through the postcloacal sensilla and spicules, by adopting an alternate, slow search behavior. We hypothesize that the reduced backing rate during this alternate behavior allows these organs, which normally act locally, to detect the vulva as the they pass it. Thus, we do not believe the alternate behavior is a novel one brought on by ablation, but is rather the expansion of a behavior normally restricted to the area around the vulva. Because males do not adopt this alternative behavior with hermaphrodites lacking vulvae, we believe that the postcloacal sensilla also acts by sensing some signal from the vulva.

Spicule Insertion

Spicule insertion is mediated by the SPD sensory neurons. We propose that these neurons sense arrival of the male at the vulva and initiate spicule protraction. Upon receiving that signal, the spicule motor neurons SPCL/R excite the spicule protractors and inhibit the spicule retractors, allowing the spicules to extend into the vulva.

Sperm Transfer

We propose that the spicule sensory neurons SPVL/R serve to inhibit the transfer of sperm until the spicules are properly situated in the vulva. Once the SPVs sense that the spicules are in the uterus, they release sperm transfer from inhibition. Ablation of the SPVs results in release of sperm outside of the vulva. Double-ablation experiments indicate that the function of the SPVs is dependent upon the function of the SPCs and SPDs. This dependence ensures spicule insertion into the vulva before release of sperm. Thus, sperm transfer is regulated not only by some signal inside the vulva acting via SPV, which signals it to release sperm transfer from inhibition, but also by a signal from the SPC neurons (direct or indirect), indicating that spicule insertion has occurred, probably via proprioceptive feedback. The dependence of SPV function upon SPD function can be explained by connection through the SPCs. In addition, another signal must be acting in response to the vulval signal to promote sperm release, as SPV-ablated animals do not release sperm constitutively in the absence of the vulva.

General Conclusions

We have been able to assign a role in male mating to all of the male-specific sensory structures and have identified structures and neurons responsible for each step of mating behavior. The sensory rays mediate both response to contact with the hermaphrodite and turning around the hermaphrodite head or tail; the hook mediates approximate location of the vulva; the postcloacal sensilla, together with the spicules, mediate precise location; and the spicules mediate spicule insertion. Each of the identified steps can be affected by elimination of 1 or more identified neurons. Thus, separable neuronal components mediate each of the steps, indicating that these steps are not identified arbitrarily. Moreover, the observed defects suggest the specific mutant phenotypes to be used for a genetic analysis of this behavior.

As analysis of invertebrate behavior proceeds, it is clear that these systems are far more complicated than originally thought. Several systems have been shown to be capable of both non-associative and associative learning (reviewed by Carew and Sahley, 1986). Analysis of central pattern-generating circuits shows that, owing to modulation, a neural network is capable of generating more than one activity pattern (reviewed by Getting, 1989; Harris-Warrick and Marder, 1991). Genetic analysis of Drosophila behavior reveals both the plasticity described above and a high degree of sensory regulation. For example, as in C. elegans, courtship behavior comprises a series of highly stereotyped acts (reviewed by Quinn and Greenspan 1984; Hall, 1994), yet it is highly dependent upon visual and olfactory cues. In addition, though these cues normally act sequentially, the sensory pathways are partially redundant, the presence of one being able to compensate for the absence of the other.

In C. elegans mating behavior, we have demonstrated that a high degree of sensory regulation also exists, such that each step is regulated by feedback from different sensory organs. Instead of a fixed motor program, this sensory feedback at each step allows the male to adjust his behavior as the environment warrants. Thus, he is capable of responding appropriately to any part of the hermaphrodite with which he comes into contact, as opposed to requiring a specific start site and then executing an invariant motor program. Should the male encounter the hermaphrodite with the ventral side of his tail, he can simply initiate backing rather than beginning the shallow ventral arching he exhibits in response to dorsal contact. Should he contact the vulva before he encounters her head or tail, he is capa-

ble of detecting a signal from the vulva and responding to it by ceasing backward motion, even if he has not executed the usual motor program for turning behavior.

We have also found that redundancy of sensory feedback allows for plasticity in behavior. The male receives information regarding vulva location, not only from the hook sensillum but also from the postcloacal sensilla and the SPD spicule sensory neurons. Information from these structures is normally used sequentially; however, in the absence of normal feedback from the hook sensillum, signaling arrival at the vulva, the male is capable of using feedback from the postcloacal sensilla to determine the location of the vulva. Behaviorally, whereas a slow search is normally seen only in the approximate area of the vulva, in the absence of the hook, the male abandons the fast search and uses a slow search to find the vulva.

Experimental Procedures

Strains and Strain Maintenance

For these studies, four strains, all isolated from N2 Bristol, were used: *him-5(e1490)* (Hodgkin et al., 1979), *unc-31(e169)* (Brenner, 1974), *unc-52(e444)* (Brenner, 1974), and *let-23(sy1)* (Aroian and Sternberg, 1991). Worms were cultured as described by Brenner (1974; Sulston and Hodgkin, 1988) at 20°C, except during behavioral observations that were made at room temperature, around 22°C–23°C.

Nomenclature

Nomenclature for C. elegans cell names is from Sulston and Horvitz (1977) and Sulston et al. (1983). Cells in a lineage are designated by a blast cell name (a combination of a capital letter and possibly a number) followed by an a, p, d, v, l, or r, standing for anterior, posterior, dorsal, ventral, left, or right, respectively. Each lower case letter denotes a cell division and refers to the position of the daughter cell. A period is used to denote the transition from embryogenesis to the larval stages, with all letters to the right of the period referring to postembryonic divisions. Terminal cells that are neurons are also referred to by a combination of three capital letters. Where symmetrical cells exist on the left and right sides, they are denoted by the letters L and R, respectively.

Cell Ablations

C. elegans is primarily hermaphroditic and gives rise to males with a frequency of only 0.2%. The strain *him-5(e1490)* (isolated from N2 Bristol; Hodgkin et al., 1979) gives rise to a higher incidence of males (33%) and has a slightly lower brood size, but is otherwise wild type. Since subsequent genetic analysis will require use of the *him-5* mutation, we chose to characterize this strain.

Males were selected from a mixed population of *him-5(e1490)* worms. The age of the animals selected depended upon the position of the targeted cell along the lineage. The animals were mounted on a glass slide for Nomarski microscopy (Sulston and Horvitz, 1977) on a 5% agar pad with 2–4 mM sodium azide (depending on age of animals) as an anesthetic. The targeted cells were ablated by focusing a laser microbeam on the nucleus of the cell, as described by Sulston and White (1980; Avery and Horvitz, 1987, 1989). Animals were recovered from the slide in M9 buffer and placed onto individual plates with bacterial lawns. A few hours later (about two cell divisions later), the worms were remounted without azide to verify that the proper cell(s) were killed and that no unintentional damage was done.

Animals were allowed to mature into young adulthood. They were then observed individually for behavioral defects in mating. Afterwards, the animals were remounted for observation under Nomarski optics to determine whether unwanted damage had occurred during the ablation procedure or some other part of handling. If damage was detected, the data collected for the animal were not used. (This number was less than 5%.) Finally, the mating efficiency of each male was determined.

Mating Efficiency Tests

This procedure was first described by Hodgkin (1983) to measure the mating efficiency of males. Each male is placed individually on a mating plate with six hermaphrodites carrying a recessive marker. The percentage of cross-progeny it sires is determined by the number of nonmarked progeny divided by the total number of progeny. For our studies, we used hermaphrodites from the marked strain *unc-52(e444)*. The strain was chosen because the mutation is easy to score, and the lack of movement facilitates mating.

Observations of Behavior

The behavioral phenotype of intact and ablated males was determined by observation with young adult *unc-31(e169)* hermaphrodites on a 0.5 cm diameter lawn of Escherichia coli (OP50) bacteria. The use of *unc-31* hermaphrodites allowed for observation with hermaphrodites that were sluggish, making it easier for the male to keep pace with the hermaphrodite. Note that this method will be sensitive to only the more severe mating defects, as males that are only slightly impaired will probably be able to mate with a sluggish hermaphrodite.

Measurements were obtained in the following manner: time was kept with a stop watch, and distances were estimated by eye and confirmed from micrographs taken of the behavior. The number of sperm transferred was determined by counting the number of cross-progeny sired from one successful mating. This method assumes that all the male sperm transferred will be utilized to generate progeny. Thus, the actual numbers of sperm transferred may be higher.

Mating behavior is sensitive to a number of variables, some known, some suspected. For example, males fail to respond to hermaphrodites if the observation plates are too dry. For that reason, plates were not used if they were more than a week old. Other suspected variables are room temperature, concentration of dauer pheromone, and hermaphrodite age. For these reasons, intact control animals, isolated at the same time as their ablated siblings, were observed under the same conditions with each round of ablations. If intact animals had difficulty mating, data for that batch of ablated animals were discarded.

Operated males were determined to be defective if they tried but failed to perform a step a total of at least ten times with at least three different hermaphrodites. The first criterion ensures reproducibility in the male's behavior; the second controls for possible difficulties with specific hermaphrodites. We found, by observation with intact animals, that not all hermaphrodites allowed easy access into the vulva. If one male failed to insert his spicules, another was likely to fail; if one succeeded, another was likely to succeed (about 85%). Thus, this high failure rate is hermaphrodite specific. Similar spicule insertion failures are seen with mating observations done with hermaphrodites of the strain *unc-52* and with wild-type hermaphrodites (*him-5*). Thus, the phenomenon is not specific to the *unc-31* strain used. To control for possible erroneous spicule insertion defect scorings, and other possible hermaphrodite-specific problems, we used at least three hermaphrodites per male. For measurements specifically concerned with spicule insertion, the hermaphrodites were preselected by the ability of intact males to insert their spicules.

Some representative records of behavioral observations are given below for ablations in the P10.p lineage, which generates the hook (Figure 4), which in turn mediates vulva location. The symbols used are as follows: r, responded to hermaphrodite contact; t, turned; v, stopped at vulva; si, inserted spicules; st, transferred sperm; p, passed vulva without stopping; h, hesitated at vulva; b, resumed backing after hesitation; ..., removed hermaphrodite; /., blocked at that step. Intact animals exhibit one of the following behavioral sequences: r/v/si/st, r/t/v/si/st, or r/t/t/v/si/st, depending upon how many times a male encounters the end of the hermaphrodite (and so must turn) before he encounters the vulva. Following ablation of P10.pppa (the HOA neuron), we observed the following sequence: r/t/p/t/t/p/t/t/p/t/t/h/t/t/p/t/t/p...r/t/t/p/t/t/ h/t/t/p/t/t/p/t/t/p...r/t/p/t/t/p/t/t/p..., which we scored as vulva location defective. In the absence of P10.ppppa (PVZ interneuron), we observed: r/t/t/v/./b/t/t/v/./b/t/t/v/...r/t/v/si/st, which we scored as having no mating defect. Ablation of P10.ppaa (HOso) gave mixed results. After ablation, one animal exhibited the following: r/t/t/h/t/t/v/b/t/t/ v/b/t/t/v/b/t...r/t/h/t/t/v/...r/t/v/si/st, which we interpreted as having no behavioral defect, while another exhibited: r/t/p/t/t/p/t/t/h/t/t/h/t/t/p/

t/t/p... r/t/t/p/t/t/p/t/t/h/t...r/t/t/p/t/t/h/t/t/h/t/t/p..., which we interpreted as vulva location defective. After ablation of P10.papp (the hook structural cell), the animal behaved as follows: r/t/v/./b/t/t/v/./b/t/t/v/... r/t/t/v/./b/t/t/p/t/t/v/...r/t/v/si/st, which we interpreted as normal behavior.

The scoring method we used addressed the question of whether the males are capable of performing a step after ablation. If a male failed to perform a step, we checked for reproducibility of that defect (at least ten times). If a male was able to perform the step, we checked only that he was able to perform that step with more than one hermaphrodite. As a result, the number of trials is lower with males that execute the step normally. Thus, while we can make conclusions as to whether an operated male is capable of performing a step in mating behavior, we cannot make any conclusions about changes in frequency of completed behavior of the males determined to be capable.

Some observations were also made with *let-23(sy1)* hermaphrodites in which 93% lack a functional vulva (Aroian and Sternberg, 1991). Individual animals were examined under Nomarski microscopy to confirm that they were vulvaless. This strain was used for some observations on ablated animals with possible vulva location defects.

Photomicrography

Behaving animals were photographed on Petri plates, through a Wild M420 macroscope, with ILFORD XP2 400 film. Exposures were taken at ASA 800. Nomarski micrographs were taken with a 100× objective.

Acknowledgments

We thank Chand Desai for suggesting this line of investigation. We thank Linda Huang, Tom Clandinin, Wendy Katz, Jing Liu, and Yvonne Cronin-Hajdu for their comments on the manuscript. Further thanks go to Mark Konishi, Gilles Laurent, David Anderson, Erin Schuman, and the two anonymous reviewers. This research was supported by the Howard Hughes Medical Institute, with which P. W. S. is an investigator, and a PYI award from the NSF to P. W. S. During part of this research, K. S. L. was a Hansen graduate fellow.

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Received August 1, 1994; revised October 4, 1994.

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Appendix:

Male inter and motor neuron ablations

Introduction

The male possesses 87 sex-specific neurons, the majority of which are associated with the copulatory structures in the posterior part of the body (Sulston et al., 1980). With the exception of the four cephalic companion cells (CEMs), the male specific cells are generated post-embryonically from a limited number of blast cells (Table 1.)

Table 1. The male copulatory structures, their associated neurons, and the male specific blast cells from which they are derived.

Structure	Neurons	n	Blast Cell
rays	RnAL/R, RnBL/R	36	V5.pL/R, V6.pL/R,
	(n = 1-9)		T.apL/R
hook	HOA, HOB	2	P10.p
post cloacal sensillae	PCAL/R, PCBL/R,	6	Y.p
	PCCL/R		B.al/rpa
spicules	SPCL/R, SPDL/R,	6	B.al/rpa
	SPVL/R		B.β

Note that all the male specific neurons are generated from a limited number of blast cells so that groups of male specific neurons can be easily ablated by killing one progenitor. (Data of Sulston et al. (1980).)

In chapter 2, the sensory neurons associated with the copulatory structures were analyzed by cellular ablation for their role in mating behavior. Here, I discuss the analysis of the remaining sex-specific neurons.

Results

For the purpose of organization, I discuss these cells by order of the blast cells from which they are derived.

B.pp

Ablation of the blast cell B.pp eliminates two inter neurons, DVE and DFV, a cell that forms part of the proctodeum, and one that forms ventral hypodermis. Ablated animals have no consistent behavioral defect but the resulting mating efficiency is drastically reduced compared to intact males (Table 2). Ablation of either DVE or DVF alone again gave no visible behavioral defects, but higher mating efficiencies than ablation of the precursors. In *plg-1* males, "plugging" efficiency was also tested and similarly reduced. The inability to sire cross progeny suggests a defect in sperm transfer. However, careful observation shows that sperm is transferred during mating, raising the possibility that the activation of sperm during transfer is under neuronal control.

Table 2. B.pp ablation results

Cell(s) Ablated	Neuron(s) Missing	ME	plug
none	none	100%	92%
B.pp	DVE, DVF + proct.	11%	20%
B.ppa	DVE + proct.	33%	40%
B.ppp	DVF + proct.	40%	36%
B.ppap	DVE	41%	28%
B.ppppa	DVF	38%	33%
B.ppap & B.ppppa	DVE, DVF	20%	18%

(The mating efficiency of intact males is 100% by definition. "plugging" efficiency was determined by crossing individual males (n=6 for each case) with 6 *unc-31*, young adult hermaphrodites for 24 hours. The number of plugged hermaphrodites was then counted. Plugging ability was consistent across *plg-1* males.)

P3.a - P8.a

These blast cells go on to form the CP(1-6) motor neurons and the CAs (1-6). Curtis Loer has done these ablations (Loer and Kenyon, 1993) and shown that the CPs are necessary for the execution of a normal turn. The CPs synapse onto the diagonal muscles which mediate the deep ventral bend in the posterior third of the male during a turn.

P9.a - P12.a

These blast cells give rise to the motor neurons CA7-9, CP7-8 and the interneuron PVX. They also give rise to the posterior motor neurons which mediate locomotion. Ablation of daughter cells, P9.aap-P12.aap, which

eliminates only the male specific neurons in question, gave no observable defects and further analysis was not pursued.

P11.p

Ablation of P11.p removes three interneurons and a motor neuron, plus three hypodermal cells associated with the hook (figure 1A). Morphologically, P11.p ablated animals appear normal except that the hook is sometimes displaced anteriorly. Males with displaced hooks stop with the hermaphrodite vulva anterior in relation to their cloaca, slowing the process of spicule insertion but not eliminating it. This is probably due to the PCS as described below. In addition, males fail to maintain backward motion during copulation. Instead, they swim forwards and away from the hermaphrodite.

Ablation of P11.pp removes only the three hypodermal cells . Again, the hook is sometimes displaced anteriorly and in these animals, the behavioral defect is consistent with an anterior hook. In contrast, ablation of P11.pa removes the four interneurons. Behaviorally, these males respond normally, initiating backing behavior, then swimming forward and away from the hermaphrodite (figure 1B). Further ablations of P11.ppa and P11.ppp etc., identified P11.ppap, otherwise designated as PVY, as the neuron primarily responsible for maintaining backing behavior. We postulate that the role of PVY is to inhibit forward motion and promote backward motion.

U, F

Because the blast cells U and F generate the same cells, four EF neurons and four DX neurons (figure 2A), we considered the two blast cells

together. Ablation of U and F in the L1 larvae resulted in males with severely crumpled spicules. This is because these cells actually serve as a signal source for cell fate determination of the spicule progenitors Bα and Bβ (Chamberlin and Sternberg, 1993). Ablation at a later time of the daughter cells, Ur/l and Fr/l, results in males with normal morphology. However, ablation of Ur/l, eliminates the killer of the linker cell, which is responsible for connecting the male gonad to the vas deferens. Behaviorally, these animals fail to turn when coming to the end of a hermaphrodite, stopping there. And of course, they do not transfer sperm. Ablation of either Ur/l or Fr/l alone eliminates the turning defect, indicating that cells generated from both lineages contribute to the behavior (probably either all four EFs or all four DXs). Ablation of Ur/lav and Fr/lvl results in males with a turning defect which also fail to transfer sperm. Thus, there is a sperm transfer defect caused by something in addition the absence of the killer of the linker cell. Ablation of Ur/lav and Fr/lvld gives the same results. Again ablation of either Ur/lav or Fr/lvl alone results in the absence of these defects.

Ablation of the DXs alone result in males which turn normally but fail to transfer sperm (figure 2). As a control, males in which the killer(s) of the linker cell had been ablated (Ur/lpd) were compared under Nomarski to males in which the four DX neurons had been ablated. While eliminating the killer of the linker cell results in males with excess quantities of sperm stored in the spermatheca (Sulston and White, 1980), DX ablated males had sperm released inappropriately into the vas deferens. In intact males, sperm is stored in the spermatheca and released into the vas deferens only upon insertion of the spicules. In DX minus worms the sperm is released prematurely. As sperm are activated upon release into the vas deferens

(Ward and Carrel, 1979), these sperm quickly lose their potency and become dark, refractile and irregular in shape. Thus we infer that the DX neurons are responsible for inhibiting sperm release into the vas deferens until spicule insertion. (Note: because Ur/lav does not always divide to give EF3 and EF4 and DX3 and DX4, some of the data were gathered as ablations of Ur/lav and either Fr/lvlda or Fr/lvldp. Thus we eliminated a pair of EFs and a pair of DX's plus an additional pair of either EFs or DXs. The behaviors observed should be the same as if the additional pair of neurons were not knocked out since the EFs and DXs are redundant, as shown by the above experiments.)

Ablation of the EFs alone resulted in animals which were defective in turning (figure 2B and figure 3B) but when successful in spicule insertion could transfer sperm. Thus the EFs are responsible for turning in intact males. This concurs with the observation that the EFs are postsynaptic to the ray sensory neurons (Sulston et al., 1980).

Combination kills: T.apl/r; EFs

Since both the T-derived rays and the EF interneurons are involved in turning behavior, we wanted to see if the EFs acted downstream of the T ray sensory neurons or in an alternative pathway. We eliminated both sets of cells by either ablating T.apr/l and then the four individual EFs or ablating Tapr/l and then U.l/ra and F.l/rv, which also eliminates the DX interneurons. Because U.l/ra do not always divide to form DX and EF, this second operation is much easier to perform. We know from ablations of the DXs alone that they play no role in turning so the additional removal of the DXs in these ablations should not affect the results. The doubly ablated animals exhibited

the same phenotype as animals in which only the EFs were ablated (figure 3C). Thus, the expression of the T ray defect requires the EF interneurons, suggesting that they are functionally connected.

Discussion

Through strategic cell ablations, we have been able to assign a role for male mating for the majority of the male specific cells studied. And we have identified neurons responsible for each step of mating behavior. The strategy has clearly been successful. However, upon looking at the cell types of the neurons that have been successfully assigned, it is evident that while sensory and motor neurons have successfully been assigned roles in this manner, few male specific interneurons have had roles assigned. While sensory and motor neurons obviously are required to mediate these steps, interneurons are expected to be involved in mediating these steps and in coordinating them. Here again, we have not encountered any cells which when ablated lead to defects in integration between steps.

To address the first question, we might look closer at the behavior in other ways. The paradigm we have developed using uncoordinated hermaphrodites is highly artificial. In normal mating, the male must keep up with a moving hermaphrodite, both backwards and forwards, and coordinate this movement with his own backward motion. Also, we could test further for possible redundancy in the system, ablating combinations of multiple cells, not only between male specific cells but those shared by the hermaphrodite as well.

Tying in the non sex specific nervous system should address the second question as well, as motor execution of these steps in behavior most likely are mediated by the general nervous system, which mediates movement in general. For example, during "backing" the male swims backwards with the posterior third of his body kept rigid against the hermaphrodite's. This backing motion is likely mediated by AVA and AVD, the command interneurons identified by Chalfie (1985), which control backward motion.

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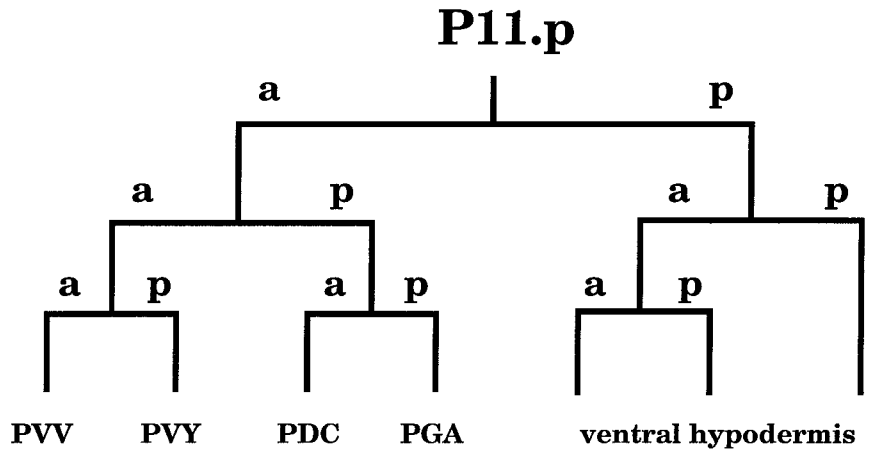
Figure 1. Analysis of the P11.p lineage

(A) The P11.p lineage, which generates three interneurons, PDC, PGA, and PVY and one motor neuron, PVV.

(B) Results of ablations of the P11.p ectoblast and its descendants.

Figure 1.

A.



B.

		Backing	
Cell Ablated	Missing	by male	by trial
none	none	10/10	98/100
P11.p	neurons &hypodermis	0/10	21/100
P11.pa	neurons	0/10	18/100
P11.pp	hypodermis	10/10	100/100
P11.paa	PVY, PVV	0/10	23/100
P11.pap	PDC, PGA	10/10	98/100
P11.paaa	PVV	10/10	97/100
P11.paap	PVY	0/10	34/100

Figure 2. Analysis of the U and F lineages

(A) The U and F lineages together produce both the 4 EF interneurons and the 4 DX motor neurons.

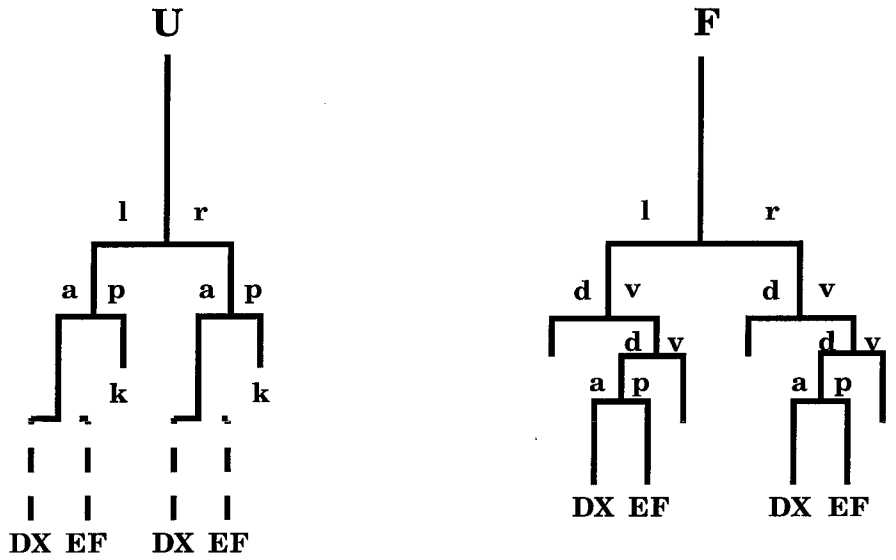
(B) Results of ablations of U and F descendants

"k" designates killer of the linker cell.

"+" indicates that additional cells were removed with this ablation.

Figure 2.

A.



B.

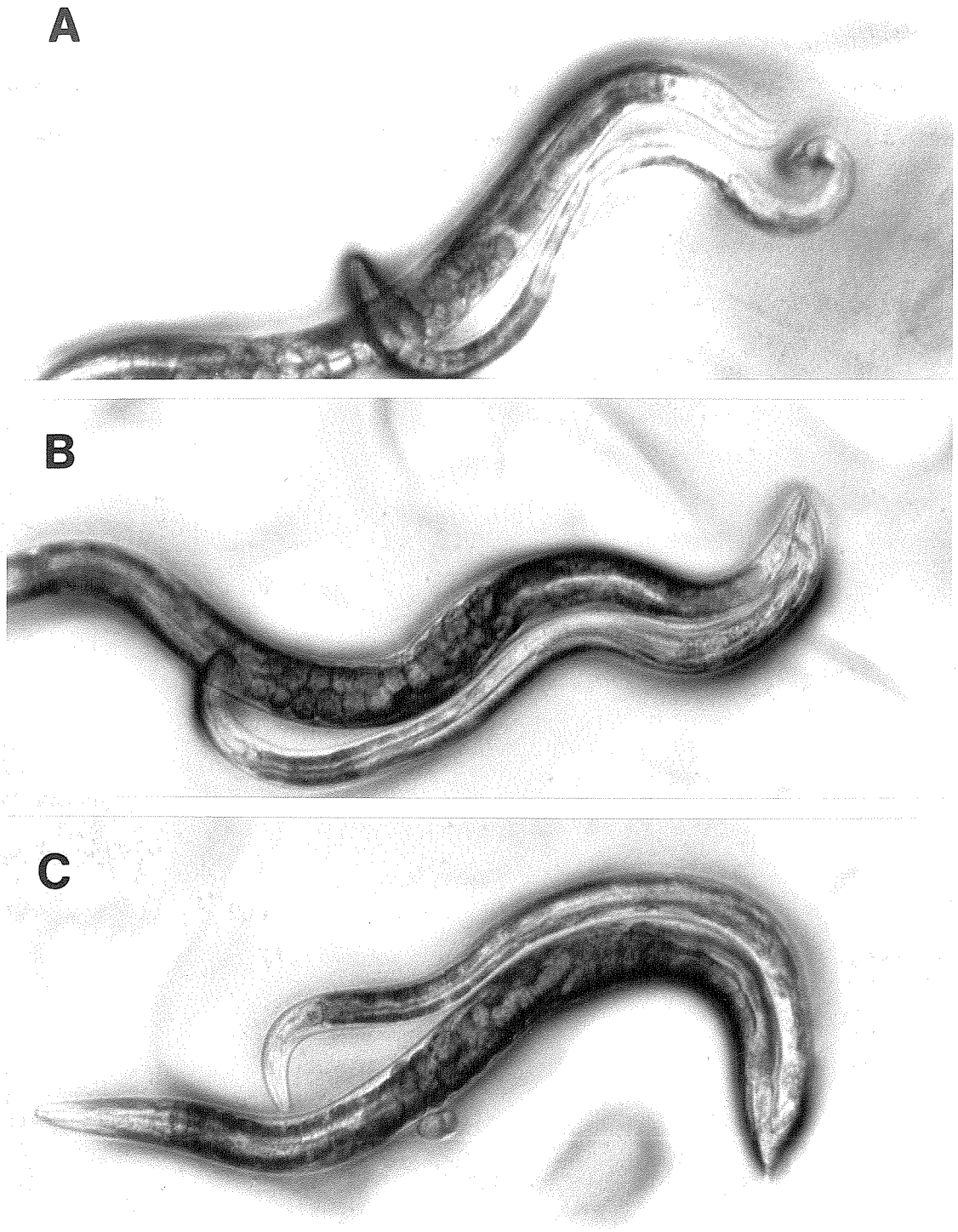
		Turning		
Ablated	Missing	by male	by trial	Sperm Defect
none	none	10/10	97/100	--
Ul/r, Fl/r	4 EFs, 4 DXs, 2 k +	0/10	0/100	loose sperm
Ul/ra, Fl/rv	4 EFs, 4 DXs +	0/10	0/100	loose sperm
Ul/ra	2 EFs, 2 DXs	10/10	97/100	--
Fl/rv	2 EFs, 2 DXs +	10/10	98/100	--
Ul/ra, Fl/rvd	EFs, DXs	0/10	0/100	loose sperm
Ul/rp	killer of linker cell	10/10	93/100	blocked sperm
Ul/ra, Fl/rvda	4 DXs, 2 EFs	10/10	76/100	loose sperm
Ul/raa, Fl/rvda	4 DXs	6/6	54/60	loose sperm
Ul/ra, Fl/rvdp	4 EFs, 2 DXs	0/10	4/100	--
Ul/rap, Fl/rvdp	4 EFs	0/4	7/100	--

Figure 3. Turning behaviors of surgically altered males

(A) Ablation of the T-derived sensory rays results in males that swim off the end of the hermaphrodite, ending in a tight ventral coil.

(B) Ablation of the EF interneurons results in males that swim to the end of the hermaphrodite and stop, failing to turn.

(C) Ablation of both the T-derived sensory rays and the EF interneurons results in males with the same behavioral defect as ablation of EF alone.


A
B
C

Chapter 3

Copulation Defective Mutants in *Caenorhabditis elegans*

Katharine S. Liu, Yvonne M. Hajdu-Cronin, and Paul W. Sternberg

ABSTRACT

Male mating behavior in *C. elegans* is a complex behavior comprising several steps: response, turning, vulva location, spicule insertion, and sperm transfer. Because *C. elegans* exists primarily as self-fertilizing hermaphrodites, mutations which eliminate mating behavior do not affect the viability of the strain. We have isolated and characterized mating defective mutants to better understand the basis of this behavior. Using two screens of different sensitivities, we have screened over 4,000 haploid genomes, isolating 42 strains defective in male mating. First, assaying the inability to sire cross progeny, we isolated 17 mutant strains, which have been mapped and tested for complementation. Three are allelic to previously identified genes: *che-3*, *fer-15*, and *goa-1*. The rest appear to define new genes. Second, using a visible copulatory plug as a marker for mating, we isolated 25 additional mutants. Five of these are defective in vulva location, a phenotype which was not detected in the first screen. We have been able to isolate mutants defective in each identified step of mating behavior. Thus, not only is mating behavior independently mutable, but each step of the behavior as well.

INTRODUCTION

The field of neurogenetics is based on the underlying premise that behavioral traits, mediated through the nervous system, are at least in part genetically determined. Thus, using behavioral defects as an assay should

allow the identification of genes that directly affect the development and function of the nervous system. One can start with a known behavioral defect to identify its neural and genetic basis. Aside from clinical neurogenetics, this is best exemplified by work done on the mouse cerebellar mutants (reviewed in CAVINESS and RAKIC, 1978). Alternatively, one can target a specific neuronal function of interest and screen for mutations based upon the predicted behavioral defect. The latter approach was pioneered in *Drosophila* (BENZER, 1973) and has been used most successfully there to uncover genes that mediate behaviors as diverse (and yet intrinsically related) as learning (DUDAI, 1985), olfaction, (SIDDIQI, 1987), biological rhythms (DUNLAP, 1993), and courtship (HALL, 1994). From these mutants, one can then begin to answer questions about nervous system development (neuronal cell fate specification or cell and axon guidance), general function (neurotransmitter synthesis and release), or even address neuronal control of the behavior itself.

The genetic tractability and simple nervous system of the nematode *C. elegans* make it particularly amenable to this latter approach. Screens for general debilitation, as evidenced by an "uncoordinated" (Unc) phenotype BRENNER, 1974), have led to the identification of genes used generally throughout the nervous system. These include genes involved in neurotransmitter synthesis (RAND and RUSSELL, 1984; McINTIRE, JORGENSEN, and HORVITZ, 1993) and axonal guidance (HEDGECOCK, CULOTTI, and HALL, 1990). Somewhat amazingly, these mutants are quite viable in the pampered environment of the laboratory Petri dish. In addition, researchers have also identified genes which appear to be more specifically involved in such behaviors as touch response (CHALFIE and SULSTON,

1981), egg-laying (DESAI, GARRIGA, MCINTIRE, and HORVITZ 1989), olfaction (BARGMANN, HARTWIEG, and HORVITZ, 1993), pharyngeal pumping (AVERY, 1993), and defecation (THOMAS, 1990; LIU and THOMAS, 1994). Many of the mutants isolated show no defects in other behaviors.

The success of these screens demonstrates that mutations can be isolated that affect very specific parts of the nervous system. We have chosen to study a relatively complex behavior in *C. elegans*: male mating behavior. Male mating behavior in *C. elegans* comprises a sequence of successive steps starting with: response to hermaphrodite, turning around her head or tail, location of the vulva, insertion of the spicules, and culminating in transfer of sperm. The success of this behavior requires the correct execution of each step and coordination between steps. While the behavior is complex, the sequence of steps is still reproducible enough that aberrations can be easily identified. As *C. elegans* exists primarily in the form of a self-fertilizing hermaphrodite, mutations that completely eliminate mating behavior are still viable.

From previous cellular analysis, we know that mating behavior is subject to a high degree of sensory regulation (LIU and STERNBERG, 1995). Initiation of and coordination between steps is controlled by a series of external sensory cues. Thus, the mutations that we expect to isolate will likely affect the interneurons that process these cues and the sensory structures that receive them.

MATERIALS AND METHODS

General methods: Worms were cultured as described by BRENNER (1974; SULSTON and HODGKIN, 1988) at 20°C, except during behavioral observations which were made at room temperature, around 22-23°C.

Mutations used:

LGI: che-1(e1034), che-3(e1124), che-13(e1805), dpy-5(e61), egl-31(n472), egl-32(n155ts), egl-33(n151ts), lin-10(e1439), unc-13(e51), unc-29(e193), unc-75(e950)

LGII: dpy-10(e128), fer-15(hc15ts), fer-15(b26), fer-15(eb1), let-23(sy1), rol-1(e91), unc-52(e444), mnDf96

LGIII: dpy-17(e164), dpy-18(e364), lon-1(e185), plg-1(e2001d), unc-25(e156), unc-32(e189), unc-49(e382), unc-93(e1500sd), nDf20

LGIV: bli-6(sc16), dpy-4(e1166sd), dpy-13(e184sd), lin-3(e1417), unc-5(e53),unc-24(e138), unc-31(e169), unc-44(e362), vab-2(e96), mnDf5

LGV: dpy-11(e224), him-5(e1490), lin-25(e156), rol-3(e754)

LGX: lon-2(e678)

Mutant isolation:

Parental strains:

C. elegans is primarily hermaphroditic, with males occurring spontaneously at a frequency of about 0.2%. Because we were concerned mainly with a male phenotype, we used *him-5(e1490)* as our parent strain. Due to increased non-disjunction of the X chromosome (HODGKIN et al., 1979), this strain gives rise to males at a frequency of 33%.

The strain *plg-1(e2001d);him-5(e1490)* was used in the second screen. The strain was created by crossing a wild isolate of *C. elegans* into *him-5(e1490)*(J. Hodgkin, pers. comm.) Males of the strain lay down a copulatory plug over the hermaphrodite vulva after successful copulation (see BARKER, 1994). The strain when we received it, segregated about 25% sterile and long animals in each generation. The brood size was lower than *him-5(e1490)* (avg. 140 vs. 260) After backcrossing the strain 10 times into *him-5(e1490)*, the strain behaved as wild type *(e1490)*.

Screen designs:

We used a clonal screen developed by HODGKIN (1983). The parental strain was subjected to EMS treatment (BRENNER 1974). 20 P0's were cloned onto separate plates. From that, pools of 10 F1 animals (larval stage 4 hermaphrodites) were picked from each P0, for a total of 200 animals. From these 20 pools, 200 F2 animals were picked onto individual plates. Because of the clonal nature of the screen, it is likely that mutants from the same F1 pool are the result of the same genetic lesion. We chose to pick ten animals per pool to minimize the risk of isolating multiple copies of the same mutation while still picking enough animals to screen through. In the first screen, F3 males were then tested for their ability to sire cross progeny with uncoordinated hermaphrodites. Of those which failed to mate, their sibling hermaphrodites were maintained for further characterization of the strain. Any gross morphological or movement defects which would account for the lack of mating were immediately discarded. Because of variability in mating, only strains in which males failed the mating assay twice were kept for further analysis.

In the second screen, using the *plg-1* strain, the plates onto which the F2 hermaphrodites were picked were scored in the F3 generation for the presence of plugs over the hermaphrodite vulvas, indicating that the males had successfully mated with their siblings. All plates with plugs were discarded. From the plates lacking plugs, mating tests were then set up as before to determine the mating efficiency of the mutant males with non-moving hermaphrodites. Those which failed to sire cross progeny were treated as in the first screen described above. Of those which did sire cross progeny, males were then examined for their mating ability by direct observation with *unc-31* hermaphrodites, looking specifically for defects in turning and vulva location. Strains in which males sired cross-progeny and mated normally with non-sibling Unc hermaphrodites were set aside as candidates for possible hermaphrodite specific defects.

In both screens, males were then screened under Nomarski microscopy for morphological defects. Only those that appeared morphologically normal and moved normally (non-Unc) were kept.

In addition, we received *bx72* as a gift from SCOTT EMMONS.

Mapping and Complementation tests:

Genetic map data, summarized in Table 1, were obtained using the standard techniques (SULSTON and HODGKIN 1988). Linkage groups were assigned based on linkage to a set of markers. The markers (via heterozygotes) were crossed into the strain in question. We then selected against the marker, meaning we picked non-marked worms from plates that segregated marked animals. Failure to mate by the next generation males indicated linkage to that marker. The markers used were: *dpy-5* on I; *dpy-10*

(or sometimes *let-23*) on II; *dpy-17* on III; *dpy-13* (or sometimes *lin-3*) on IV; *dpy-11* on LGV; and *lon-2* on X.

Further two and three factor crosses (TRENT, TSUNG, and HORVITZ 1983) and deficiency mapping were done to narrow the locations of the mutations.

For all mutations which mapped to the same region as another Cod or other previously identified genes that could account for the defect, complementation tests were done to determine if they were allelic. Heterozygous (no Cods were found on LGX) males were crossed into homozygous hermaphrodites, carrying a recessive dpy marker. Unmarked male cross-progeny were tested for mating ability with the standard mating efficiency test (Hodgkin, 1983; see below). By this, *sy194* was determined to be allele of *fer-15* and *sy172*, an allele of *che-3* and *sy192* an allele of *goa-1*. All Cods complemented between themselves, indicating they were all separate loci.

Mutant analysis:

Nomarski

Candidate strains were first viewed under Nomarski optics (SULSTON and HORVITZ, 1977) at 100x for their overall morphology. In particular, we checked to see that the copulatory structures and gonad appeared normal. After the strains had been characterized for their behavioral defects, some strains were checked for the generation of neurons that were possibly involved.

FITC staining:

Mutants were assayed for their ability to take up FITC (5-fluorescein isothiocyanate) dye, an indication of sensory neuron function, using the protocol first described by HEDGECOCK, CULOTTI, THOMSON, and PERKINS (1985), 75λ of a stock FITC solution (20 mg/ml dissolved in DMF) was diluted in 300λ of M9 buffer solution, then added to a 5cm plate for two hours. (Final concentration of FITC: 0.1mg/ml) Worms were placed on the plates for two hours, then transferred to clean plates for half an hour for destain. Worms were then mounted as for Nomarski and viewed under epifluorescence, using a fluorescein filter.

muscle:

Animals were also examined under polarized light microscopy (WATERSON, THOMSON, and BRENNER, 1980) for possible defects in muscle structure.

pharmacology:

Strains were checked for their responsiveness to serotonin and imipramine based on the assays developed in TRENT, TSUNG, and HORVITZ (1983) and specifically adapted for males in LOER and KENYON (1993). Serotonin (obtained from Sigma) was dissolved in M9 buffer (SULSTON and HODGKIN 1988) to make a 20 mM solution. For the assay, the males were placed in wells containing the solution and observed individually through the dissecting microscope for 10 seconds each. Males exhibiting a tight ventral tail curl for ≥ 5 seconds were scored as responsive.

Strains were checked for their responsiveness to the GABA agonist, muscimol, based on the assay developed by MCINTIRE, JORGENSEN, and HORVITZ (1993). Males placed on a plate seeded with 10 mM muscimol were scored after 30 minutes for spicule protraction.

Behavioral analysis:

egg-laying and brood size:

Hermaphrodites were placed individually, one to a plate, checked each day for three days to see if bloated/lay eggs. Each day, the hermaphrodite was transferred to a fresh plate. The cumulative number of progeny produced by each hermaphrodite was counted.

osmotic avoidance

Strains were tested for their avoidance of high osmolarity (CULOTTI and RUSSELL, 1978). A ring of 4M fructose, with Congo red for visibility, was applied to an unseeded Petri plate. The open end of a blue micro pipette tip from Fischer was used to make the ring. We first tested its effectiveness with wild-type animals. If they failed to pass through, we used the same ring immediately afterwards to test animals of the strain in question. After the trial, the ring was again tested with wild-type animals.

touch response, pharyngeal pumping, and defecation:

Strains were tested for their response to light touch by stroking their heads and tails with a mounted eyelash (CHALFIE and SULSTON, 1981) Pharyngeal pumping rates were assayed by counting under a dissecting scope and compared to the wild-type rate of 180 pumps / minute (AVERY, 1993). The strains were checked for distended lumen and a normal defecation periodicity of about 50 seconds (THOMAS, 1990).

mating behavior:

Mutant males were examined in a manner similar to operated males in LIU and STERNBERG (1995). (See Experimental Procedures there for all the controls and caveats.) The behavioral phenotype of the strain in question was determined by individual observation with young adult *unc-31(e169)*

hermaphrodites on a 0.5 cm diameter lawn of *E. coli* (OP50) bacteria. The use of *unc-31* hermaphrodites allowed for observation with hermaphrodites that were sluggish, making it easier for the male to keep pace with the hermaphrodite. Mutant males were determined to be defective if they tried but failed to perform a step a total of at least ten times with at least three different hermaphrodites. The males were then examined under Nomarski optics to ensure that no injuries had occurred during the handling of the male that would account for any defects in mating. Where injuries did occur (<1%), the trial was discarded. Mating efficiency tests were then set up for each animal.

Mating efficiency tests:

This procedure was first described by HODGKIN (1983) to measure the mating efficiency of males. Each male is placed individually on a mating plate with six hermaphrodites carrying a recessive marker. The percentage of cross progeny it sires is determined by the number of non-marked progeny divided by the total number of progeny. For our studies, we used hermaphrodites from the marked strain *unc-52(e444)*. The strain was chosen because the mutation is easy to score and the lack of movement facilitates mating.

Photomicrography: Behaving animals were photographed on Petri plates, through a Wild M420 macroscope, with ILFORD XP2 400 film. Exposures were taken at ASA 800.

RESULTS

Isolation of mutants:

The initial screen assayed for the inability of males to sire cross progeny with uncoordinated (Unc) hermaphrodites. We chose uncoordinated partners in order to detect only the most severe defects. In this way, we hoped to avoid analyzing mutants with general defects that might impair mating behavior. After surveying a little over 2,000 haploid genomes, about 75 candidates were chosen for further analysis based on their inability (or reduced ability) to sire cross progeny and the lack of any obvious general defects that would account for it. Only 25% of these back crossed successfully for a total of 17 strains. The high degree of false positives is expected as a number of nonspecific factors can adversely affect mating behavior.

The strains have been assigned to linkage groups, further mapped (Table 1), and the appropriate complementation tests done. One of these strains appears to be a synthetic mutation involving two separate loci. The 17 mutations are distributed on all five autosomal linkage groups (Figure 1). None were found on X. Three of the mutants were alleles of previously identified genes: *che-3(sy172)*, *goa-1(sy192)*, and *fer-15(sy194)*. We had previously observed alleles of *che-3* for defects in mating behavior (appendix, this chapter) and knew that *che-3* is required for male response to hermaphrodites. Thus, it was not surprising to isolate an allele of *che-3* in our screen. While *fer-15* affects the fertility of sperm, this particular strain seems to have a behavioral defect independent of its fertility defect. *sy192*, is a loss of function allele of *goa-1* which encodes the α subunit of G_o. The

mutant has multiple behavioral defects and is characterized elsewhere (MENDEL, KORSWAGEN, LIU, HAJDU-CRONIN, SIMON, PLASTERK, and STERNBERG 1995). The remaining mutations appear to define new genes; we call these Cod for copulation defective.

Candidate Cod strains were examined for specific defects in male mating behavior which might explain their inability to sire cross-progeny (Table 2). The majority of mutants (9/17) are defective in spicule insertion (Table 2). None were found to be defective in vulva location. Previous ablation studies had shown that while the hook is the primary mediator of vulva location, the post cloacal sensillae are also capable of this role, but at reduced efficiency (LIU and STERNBERG, 1995). The existence of redundant sensory systems could explain why we did not recover any mutants in this step. Based on this hypothesis, we predicted that if we increased the difficulty encountered by males during mating, we should be able to detect mutants with defects similar to those seen in hook-ablated males. Accordingly, we devised a second screen, based on the ability of males to mate with their moving sibling hermaphrodites. To do this, we relied on a strain, *plg-1(2001d)* in which males lay down a "plug" over the hermaphrodite vulva after successful copulation. We then screened for the lack of plugs.

After screening 2200 haploid genomes, about 250 candidates were identified which failed to form mating plugs. Of the 110 candidates selected for further analysis, 25 were successfully backcrossed, again about 25%. The mutants from the second screen have been observed for their mating behavior defects but other aspects have not been fully characterized. With the exception of those defective in vulva location, these mutants will therefore not be discussed in the rest of the chapter. The number of mutants found in each

category is listed (Table 3) for comparison. The change in paradigm did allow us to detect 5 mutants defective in vulva location, as predicted. In addition, as both males and their hermaphrodite siblings were now required for successful mating, we were able to detect two strains with apparently hermaphrodite specific mating defects. These are discussed elsewhere (chapter 4). Otherwise, the numbers between the two screens are comparable. Thus, the addition of *plg-1* into the background does not affect the screen.

Analysis of mutants

In addition to observations of mating behavior, Cod strains were subject to a battery of other behavioral and morphological assays. These assays were intended to screen out mating defects caused by "nonbehavioral" reasons and, for those strains which were determined to be behavioral mutants, to provide clues as to what the underlying causes might be.

egg-laying and brood size:

Each strain was checked for hermaphrodite egg-laying behavior and brood size (Table 2). Brood size was checked to ensure that the inability to sire cross progeny was not due to a general defect in sperm fertility. This assay does not take into account male specific fertility defects (we also checked for abnormal sperm morphology in the male gonad). Egg-laying defects can result from a number of reasons such as defects in vulval development, muscle function, and the HSNs (hermaphrodite specific

neurons) which control egg-laying behavior. Due to common genetic control of some aspects of the sex-specific development, these hermaphrodite defects are often accompanied by a corresponding defect in the male. *sem-(sy35)* hermaphrodites are egg-laying defective, and we later determined that both the male turning defect and the hermaphrodite egg-laying defect are due to abnormal development of the sex-specific muscles (see "turning" below).

sensory and motor:

Cod animals were also checked for both sensory and muscle defects. The accumulation of 5-fluorescein isothiocyanate dye (FITC) in the neurons of the amphid sensilla is an indicator of proper chemosensory function (PERKINS, HEDGECOCK, THOMPSON, and CULOTTI, 1986). As many steps in mating behavior are mediated by what appear to be chemosensory organs, we expected to isolate some mutants defective in chemosensation in our screen.

As we discarded mutants which displayed uncoordinated locomotion, we did not expect to isolate mutants with general muscle defects. However, males possess several sex-specific muscles which can be affected independently of general body wall muscle. Animals were therefore examined specifically for defects in the sex-specific muscles. Of the 17 strains characterized, only two were found to have muscle defects (Table 2), both in male-specific muscles. Based on this negative evidence, we suggest that our screen is relatively specifically detecting neuronal defects.

other behaviors:

One might expect genes which control the nervous system to affect a number of behaviors, even if originally identified for their role in one. Therefore, all Cod mutants were checked for defects in other behaviors: pharyngeal pumping, defecation, response to touch, and egg-laying (see above). With the exception of egg-laying, all Cod strains were normal in these behaviors.

Mating behavior:

response:

Three strains were defective in response to contact with hermaphrodites: *cod-10(sy38)*, *che-3(sy172)*, and *sy195*. An additional response defective strain, *bx72*, was given to us by Scott Emmons.

Response to hermaphrodite contact is mediated by the sensory rays, which are most likely chemosensory (SULSTON, ALBERTSON, and THOMSON, 1980; LIU and STERNBERG, 1995). Accordingly, most of our response defective mutants are also affected in the amphid sensilla as evidenced by their failure to accumulate FITC dye (Figure 2).

cod-10(sy38) is weakly defective in response, failing to respond to contact about 65% of the time (Table 2). Correspondingly, it stains weakly for FITC.

che-3(sy172) and *sy195* both fail to respond and take up FITC. *sy172* maps in the same interval with and fails to complement alleles of *che-3*. *che-3*

had been shown previously to eliminate response to contact with hermaphrodites. Thus, *che-3(sy172)* affects response behavior via the disruption of chemosensation in general. Given the correspondence between lack of response to hermaphrodite contact and FITC uptake, we believe all three mutants discussed are mating defective due to a general defect in chemosensation.

In contrast, *bx72* is strongly defective for response yet, unlike the others, is wild-type in staining for FITC dye. Thus, this mutation may specifically affect response. To explore this possibility, we assayed *bx72* and the other response defective males for osmotic avoidance behavior. Wild-type worms will not swim through a ring of 4M fructose (CULOTTI and RUSSELL, 1975). Most amphid defective mutants do. Both *che-3(sy172)* and *sy195* were defective in osmotic avoidance, while *cod-10(sy38)* and *bx72* were not (n=10), further suggesting that *bx72* is response defective specific.

turning:

Two mutants were isolated which were defective in turning, *sem-(sy35)* and *cod-5(sy181)*, though their behavioral defects are phenotypically distinct. Wild type males turn before they reach the end of the hermaphrodite in a tight ventral flexion of their tail (Figure 3a). *sem-(sy35)* mutants swim to the end of the hermaphrodite and stop, failing to turn (Figure 3b). This phenotype looks identical to the turning defect exhibited by males in which the EF interneurons have been ablated (chapter 2). *cod-5(sy181)* males swim off the end of the hermaphrodite, ending in a "fish hook" position. Occasionally, these males succeed in turning to the other side of the

hermaphrodite but do so in a loose coil (Figure 3c). The phenotype of *cod-5(sy181)* is similar, but not identical, to the turning defect seen after ablation of the posterior sensory rays (chapter 2).

Turning behavior is executed by the male-specific diagonal muscles (Loer and Kenyon, 1993), which get their name from their characteristic diagonal alignment with respect to the body (Figure 4a). Therefore, both mutants were examined for defects in these muscles. *cod-5(sy181)* was normal with respect to both diagonal and body wall muscle, suggesting that the basis for the turning defect is neuronal. In contrast, the diagonal muscles in *sem-(sy35)* males were grossly disorganized compared to wild type (Figure 4b). Further analysis revealed that the disorganization is due a migration defect in the sex-specific myoblasts. (*sem-(sy35)* hermaphrodites are also affected, causing a defect in egg-laying behavior.) For this reason, we have named *sy35* a *sem* mutant (for *se*x myoblast *m*igration), in accordance with the current nomenclature.

Loer and Kenyon have reported that ablation of the diagonal muscles resulted in males which were unable to turn normally but occasionally did so in loose, "sloppy" arches (1993). The results suggest that, while the diagonal muscles are the primary executors of turning behavior, additional (body wall) muscles are simultaneously activated. *sem-(sy35)* males fail to turn altogether. The disorganization of the diagonal muscles suggests that, when activated, they not only fail to execute a ventral flexion but antagonize the effect of other muscles as well.

Bath application of 20 mM solution 5-HT produces a characteristic tail curl in wild-type males (Loer and Kenyon, 1993). Both *sy35* and *sy181* were assayed for this response. As expected, *sy35* males failed to curl their tails

while *sy181* males did. The "rescue" of the *sy181* phenotype by exogenous serotonin is further evidence that the defect is neuronal in basis.

vulva location:

Five strains defective in vulva location were isolated in the second screen: *sy419*, *sy420*, *sy421*, *sy422*, and *sy423*. They have been assigned to linkage groups and their behavioral defect characterized (Table 4). Ablation of the hook sensilla results in males which fail to recognize the vulva, passing it repeatedly (LIU and STERNBERG, 1995). None of the mutants isolated show defects in vulva location as severe as is caused by ablation of the hook sensilla. All males are capable of finding the vulva of Unc hermaphrodites, as evidenced by their normal mating efficiencies. Of these, *sy420* has the most severe defect, passing the vulva 80% of the time.

Males of the strain *sy419* actually find the vulva at almost wild-type frequencies but tend to lose it during the adjustive movements that accompany spicule insertion. This phenotype looks identical to that exhibited by males after ablation of the post cloacal sensillae (LIU and STERBERG, 1995).

Males of all vulval location defective strains have been examined under Nomarski for defects in hook and post cloacal sensillae structure and neuron number (n=5). All were found to be morphologically wild-type in these respects. However, neurons were identified by placement alone; other aspects of neuronal identity could not be easily verified.

The results suggest two things. Defects resembling those seen after hook ablations and post cloacal ablations can be independently phenocopied

by mutation, suggesting that their development and function are genetically separable. Second, the simplest interpretation of the mutant phenotypes of *sy420*, *sy421*, *sy422*, and *sy423* is that they impair function of the hook sensilla, but only partially. Thus, it may not be possible to selectively eliminate hook sensilla function. Mutations that do so may affect other aspects of behavior, such as response to contact with hermaphrodites, or even be lethal. However, the number of genomes screened is still small, so such a conclusion is likely premature.

spicule insertion:

Nine strains are defective in spicule insertion, including one strain that carries mutations in two loci: *cod-2(sy43)*, *cod-3(sy166)*, *cod-4(sy180)*, *cod-6(sy186)*, *cod-7(sy190)*, *cod-1(sy193)*, *fer-15(sy194)*, *sy158*, and *cod-8(sy176); cod-9(sy226)*. Neither *cod-8(sy176)* nor *cod-9(sy226)* alone are mating defective, suggesting that the defect in spicule insertion is synergistic.

sy194 fails to complement alleles of *fer-15* on the basis of mating efficiency. Mutations of *fer-15* affect sperm fertility, causing males to have reduced or no mating efficiencies. However, the spicule insertion defect of *sy194* appears to be independent of its fertility defect.

Spicule movement is controlled by two sets of muscles. Each spicule is controlled by two protractors and two retractors (SULSTON, ALBERTSON, and THOMSON, 1980). The spicule insertion defective strains were examined for spicule muscle defects under polarized light. All appear wild-type in muscle morphology with the exception of *sy43*. *sy43* males possess

additional ectopic muscles in the region of the spicule protractors, which probably account for their spicule insertion defects.

sperm transfer:

Two strains were found to defective in the transfer of sperm , *sy156* and *goa-1(sy192)*. The defect in *goa-1(sy192)* is probably due to decreased fertility as hermaphrodite fertility is affected (MENDEL, KORSWAGEN, LIU, HAJDU-CRONIN, SIMON, PLASTERK, and STERNBERG 1995).

DISCUSSION

Mutants were identified for each step of mating behavior: By screening directly for mutants in which males either failed to sire cross progeny or failed to deposit a copulatory plug over their sibling hermaphrodites, we were able to isolate mutants that specifically affect mating behavior. Several of the mutants isolated have other phenotypes due to their defects: *sem-(sy35)* hermaphrodites are egg-laying defective due to an analogous defect in sex myoblast migration; the response defects in *che-3(sy172)*, *cod-10(sy38)*, and *sy195* males are probably due to a general defect in chemosensation; *fer-15(sy194)* has a fertility defect which is tightly linked to the observed defect in spicule insertion; and *goa-1(sy192)* males suffer multiple behavioral defects due to loss in the function of Go_{α}. However, no pleiotropic effects have been found in the remaining 12 mutants that have been fully characterized. Thus, mating behavior is genetically separable,

implying that there are components specific to this behavior that mediate it. This is significant given the number of actions involved in a successful act of copulation. Moreover, each step of the behavior appears to be separable, and thus likely mediated by different systems.

Most of the observed phenotypes are probably due to neuronal defects: Response to hermaphrodites and vulva location both essentially involve the cessation of one act to begin another (backing and spicule insertion, respectively). Thus, failure to perform these steps is unlikely to be due to a muscle defect and most likely due to a sensory failure (as in *che-3(sy172)* and *sy195*) or a defect in the coordination of motor systems.

In contrast, defects in turning and spicule insertion could easily be due to muscle. Of the two turning mutants, *sem-(sy35)* males have aberrant diagonal muscles which are used in normal turning behavior. *cod-5(sy181)* has no visible muscle abnormalities. The behavioral phenotype of these males resembles the turning defect seen following ablation of the posterior sensory rays. *cod-5(sy181)* males respond to bath treatment with serotonin by curling their tails. All of this suggests that the turning defect is due to a problem in the nervous system, probably in the sensory neurons.

cod-2(sy43) males possess ectopic muscles in the area of the spicule protractors. Of the 8 remaining mutants defective in spicule insertion, at least some and likely many are deficient in some aspect of the neural function.

Response to hermaphrodites is mediated by a chemosensory mechanism: Of the four mutant strains in which males fail to respond to contact with hermaphrodites, three (*che-3(sy172)*, *cod-10(sy38)*, and *sy195*) are likely defective in chemosensation in general, indicating that the two

behaviors share a genetic basis. The most straightforward interpretation is that response is mediated by chemosensation. These three mutants are all defective in function of the amphid and phasmid sensillae, which are known to be chemosensory (DAVIS, GOODE, and DUSENBERY, 1986). Thus, it could be that response is mediated by these organs. However, ablation analysis has shown that the male specific sensory rays are necessary to mediate response (LIU and STERNBERG, 1995). Moreover, the response defective mutant *bx72* is wild-type in both FITC uptake and osmotic avoidance, suggesting that its defect is specific to mating behavior. *bx72* could affect ray sensory function or interneurons that act down stream of the ray neurons.

Vulva location is mediated by redundant and/or essential mechanisms: Our first screen, in which we assayed for the inability to sire cross progeny with uncoordinated hermaphrodites, failed to detect any mutants that were defective in vulva location. Results from our ablation analyses suggested that this was due to a redundancy in the sensory mechanisms involved in this step. Both the hook sensilla and the post cloacal sensillae are sufficient to mediate vulva location (LIU and STERNBERG, 1995). A second, more sensitive screen allowed us to isolate five mutants that are defective in vulva location. The phenotype of one, *sy419,* resembles the defect seen following ablation of the post cloacal sensillae. The remaining four, *sy420*, *sy421*, *sy422*, and *sy423*, have defects similar to that seen after ablation of the hook. Thus, the two steps in vulva location are genetically separable. However, none of the latter four strains exhibit defects as severe as those seen following ablation of the hook. This observation either implies further redundancy or that the genes involved are essential for other

functions, defects in which preclude expression of the vulva location defect. Construction of double mutants between the different vulva location defective strains should be informative in this regard.

In conclusion, we have been able to isolate mutants that appear to be specifically defective in mating behavior. Many of these are likely to have defects in the nervous system. The phenotypes of most of these mutants resemble behavioral defects seen following ablation of different male specific sensory structures used in copulation and hence, at least some of these mutations will likely turn out to be defective in these identified structures. In addition, some may define sites of action not identified by the lesion studies.

ACKNOWLEDGMENTS

LESLIE BARBER started the COD screen, isolating three which are described here. HELEN CHAMBERLIN helped with the morphological characterization of the first screen. WILL BOORSTEIN identified the muscle defects in *sy35* and *sy43*. GREG JONGEWARD and CHRISTOPHER SCHOENHERR encouraged us to pursue the second (plg) screen. ANN CHEN helped with the second (plg) screen. SCOTT EMMONS kindly provided *bx72*. Many strains used in this work were obtained from the CGC, which does not get enough credit for how much easier they have made science in the worm community. This research was supported by the Howard Hughes Medical Institute, with which PWS is an investigator, and a PYI award from the NSF to PWS. KSL was a Hansen graduate fellow during part of this research.

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Table 1. Three factor mapping data

Mutant	Heterozygote	Recombinant phen. selected	# cod/+ : # total
sem-(sy35)	dpy-5 unc-29 +/+ + sy35	Dpy	12/12 Egl/+
	unc-29 + unc-75/+ sy35 +	Unc	4/13 Egl/+
cod-10(sy38)	lin-10 unc-75 +/+ + sy38 *not under hIn1[h1040]*	Lin	10/10, 14/16
cod-2(sy43)	lin-10 unc-75 +/+ + sy43 *under hIn1[h1040]*	Lin	11/11
sy156	+ dpy-13 unc-24/sy156 + +	Dpy	1/2
	unc-17 + dpy-13/+ sy156 +	Dpy	1/1, 1/1, 0/1
	unc-44 lin-3/sy156	Lin	3/9
cod-3(sy166)	vab-2 + unc-44/+ sy166 +	Vab	4/5
	unc-17 dpy-13 +/+ + sy166	Dpy	0/2
	dpy-13 + unc-24/+ sy166 +	Dpy	7/8
	dpy-13 (+ unc-44)/+ (sy166 +)	Dpy	3/3, 8/9
	unc-44 (+ lin-3)/+ (sy166 +)	Lin	11/13
	bli-6 unc-24/sy166	Bli	1/1
	unc-5 bli-6/sy166	Bli	0/2

Table 1. Three factor mapping data cont.

Mutant	Heterozygote	Recombinant phen. selected	# cod/+ : # total
che-3(sy172)	+ lin-10 unc-75/sy172 + +	Lin	0/16
	dpy-5 + lin-10/+ sy172 +	Lin	0/8
	unc-13 + lin-10/+ sy172 +	Lin	5/10
cod-4(sy180)	+ dpy-17 unc-49/sy180 + +	Dpy	0/6
		Unc	5/5
	unc-93 dpy-17 +/+ + sy180	Dpy	0/6
	dpy-17 + unc-32/+ sy180 +	Dpy	1/20
cod-5(sy181)	+ let-23 rol-1/sy181 + +	Let (Vul)	0/6
	dpy-10 + let-23/+ sy181 +	Let (Vul)	2/2, 9/10
cod-6(sy186)	dpy-17 + unc-49/+ sy186 +	Dpy	2/11, 1/ 9
		Unc	5/7
	dpy-17 + unc-32/+ sy186 +	Dpy	7/11
cod-7(sy190)	+ lin-10 unc-75/sy190 + +	Lin	1/10
	dpy-5 + lin-10/+ sy190 +	Lin	0/14
	unc-13 lin-10/sy190	Lin	0/1

Table 1. Three factor mapping data cont.

Mutant	Heterozygote	Recombinant phen. selected	# cod/+ : # total
goa-1(sy192)	+ lin-10 unc-75/sy192	Lin	0/7 cod+loopy
	dpy-5 + lin-10/+ sy192 +	Lin	1/4 cod+loopy
cod-1(sy193)	dpy-11 + lin-25/+ sy193 +	Dpy	2/12
	dpy-11 rol-3 +/+ + sy193	Dpy	7/7, 2/2
fer-15(sy194)	+ let-23 rol-1/sy194 + +	Let (Vul)	0/6
	dpy-10 + let-23/+ sy194 +	Let (Vul)	6/9

Table 2. Behavioral analysis

Mutant	Egl	FITC	muscle	mating defect by male	mating defect by trial
response					
*cod-10(sy38)*I	+	+/-	+	7/10	34/100
*che-3(sy172)*I	+	--	+	0/10	0/100
sy195	+	--	+	0/10	0/100
*bx72*IV	+	+	n.d.	0/10	0/10
turning					
*sem-(sy35)*I	--	+	--	0/10	0/100
*cod-5(sy181)*II	+	+	+	0/10	22/100
spicule insertion					
*cod-1(sy193)*V	+	+	+	0/10	0/100
*cod-2(sy43)*I	+	+	--	0/10	0/100

Table 2. Behavioral analysis cont.

Mutant	Egl	FITC	muscle	mating defect by male	mating defect by trial
spicule insertion					
*cod-3(sy166)*IV	+	+	+	0/10	0/100
*cod-4(sy180)*III	+	+	+	0/10	0/100
*cod-6(sy186)*III	+	+	+	0/10	0/100
*cod-7(sy190)*I	+	+	+	0/10	0/100
*cod-8(sy176)*III; *cod-9(sy226)*IV	+	+	+	0/10	0/100
*fer-15(sy194)*II	+	+	+	4/10	8/100
sy-158 III;IV	+	+	+	0/10	0/100
sperm transfer					
*sy156*IV	+	+	+	0/10	0/100

Table 3. A comparison of the two screens used

Type of mutant	Number of mutants isolated	
	First screen	Second screen
response	3	2
turning	2	2
vulva location	0	5
spicule insertion	9	13
sperm transfer	2**	3
hermaphrodite specific	--	2

***sy192* was initially isolated as a sperm transfer defective mutant but turns out to have multiple behavioral defects, including turning and spicule insertion.

Table 4. Vulva location defective mutants

name	ME	% misses
wild type	3	1%
*sy419*II	3	5%
sy420 III	1	80%
*sy421*IV	2	40%
*sy422*IV	2	65%
*sy423*I	2	60%

Figure 1. Map positions

The Cods are distributed throughout the five autosomal linkage groups. No Cods were found on X.

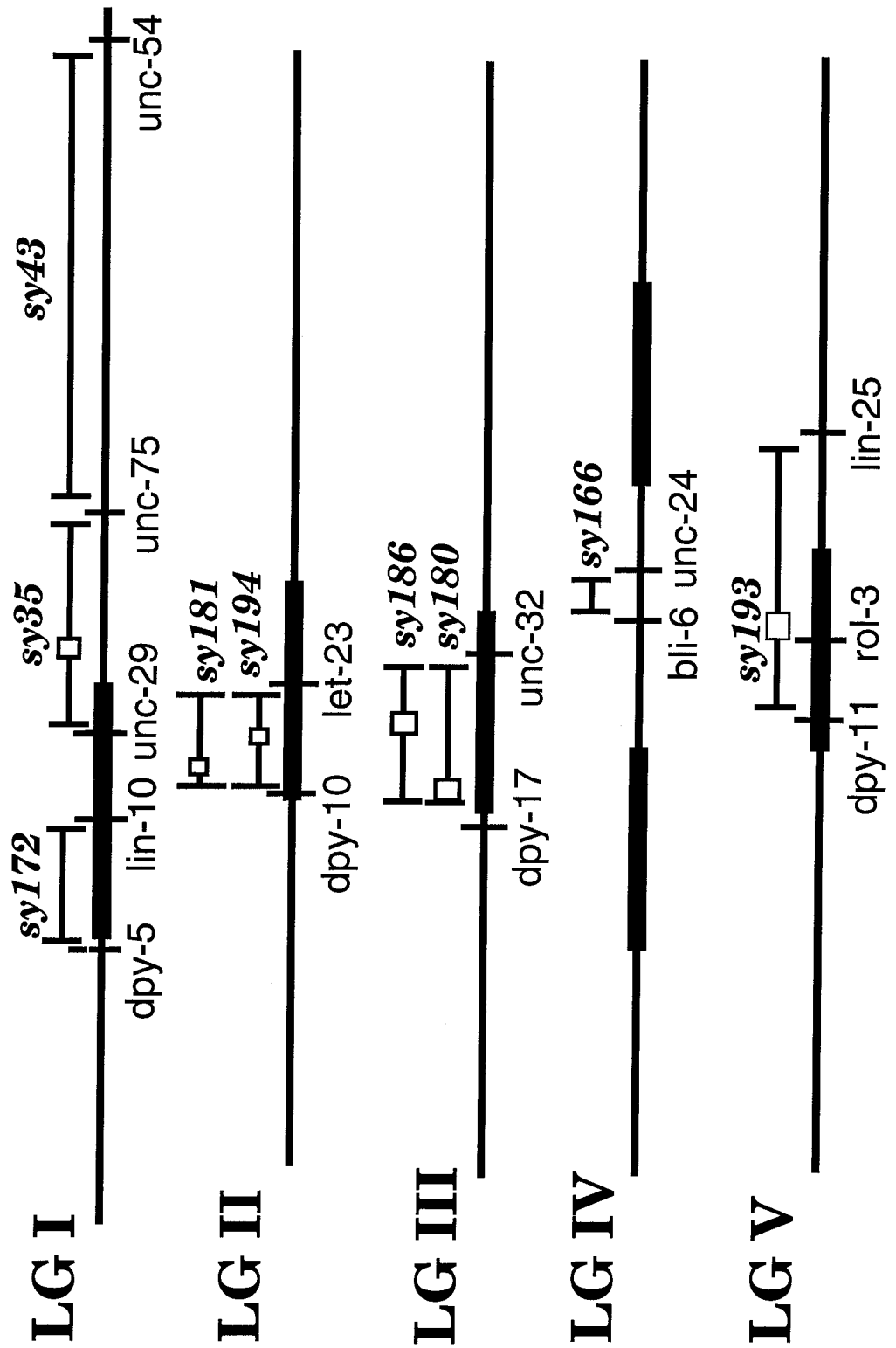


Figure 1. map positions

Figure 2. FITC staining of the amphid sensillae

(a) Wild-type: the amphid cell bodies and processes can be seen.

(b) *sy172*, a response defective strain, fails to take up FITC.

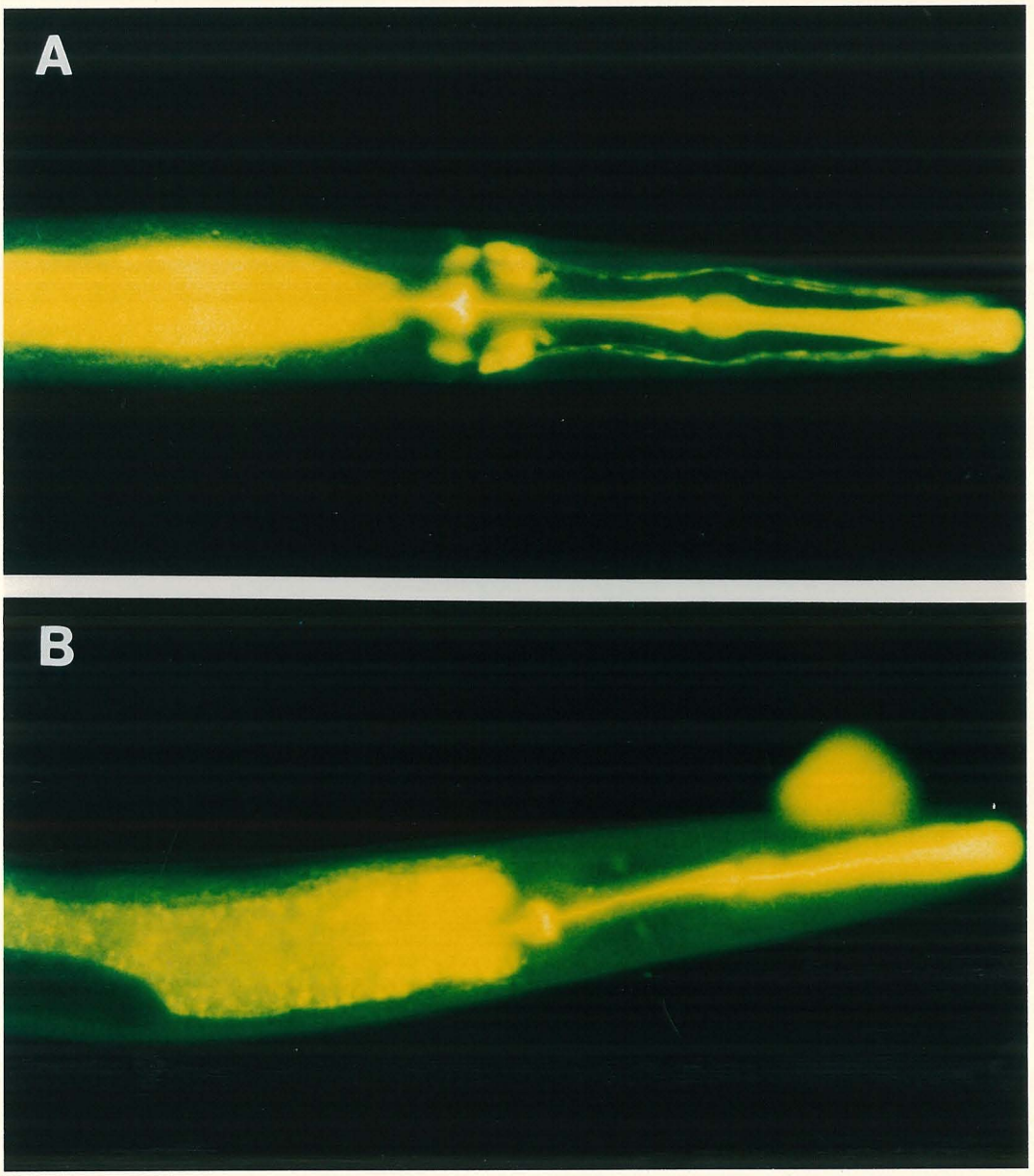

A
B

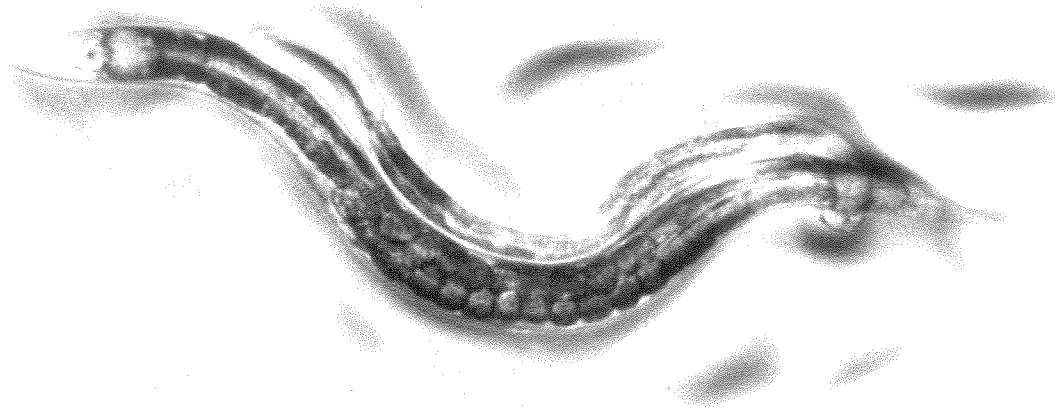
Figure 3. Comparison of turning defects

(a) Wild-type animals turn in tight ventral flexion before they reach the end of the hermaphrodite.

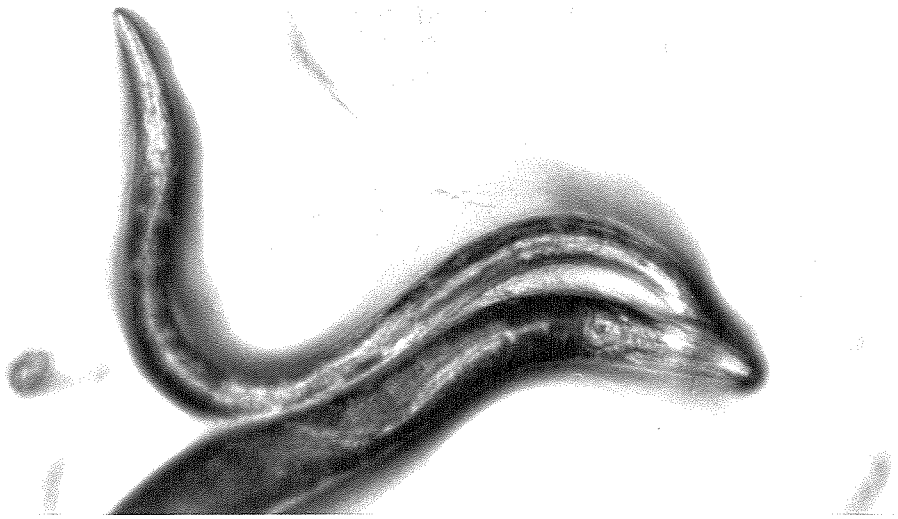
(b) *sy35* animals swim to the end of the hermaphrodite, failing to initiate ventral flexion.

(c) *sy181* animals swim off the end of the hermaphrodite, occasionally turning via a loose ventral arch.

A



B



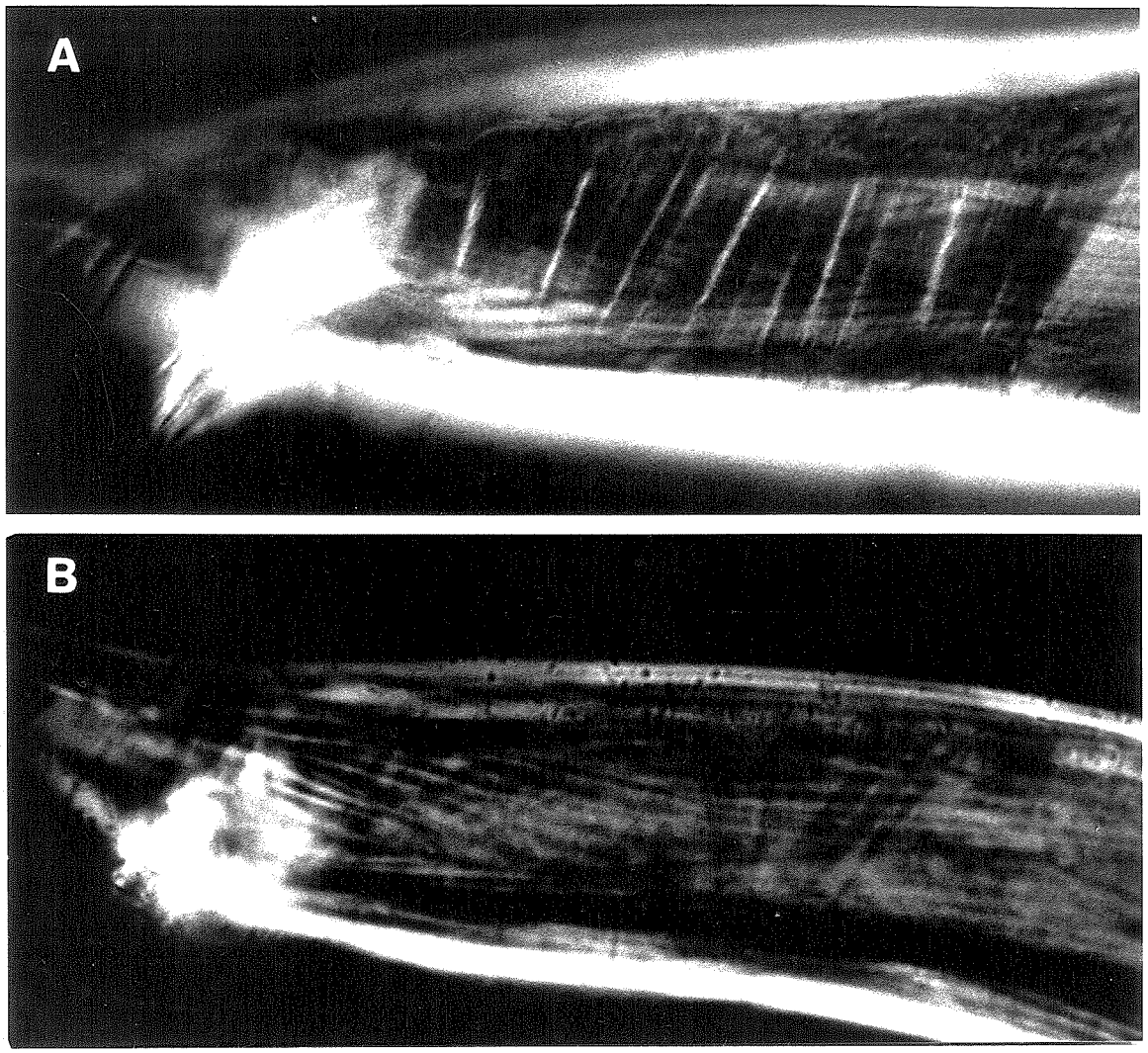
C

Figure 4. The diagonal muscles execute turning behavior

(a) The diagonal muscles in wild-type males are ordered in a diagonal angle relative to the rest of the body.

(b) The diagonal muscles of *sy35* males are randomly oriented compared to wild type.

Photographs taken by Will Boorstein.


A
B

Appendix:

Characterization of previously identified genes

Introduction

In addition to the mutants specifically isolated for copulation defects, I also characterized previously identified genes for possible defects in male mating behavior. The "screening" of pre-identified genes has proved useful in detecting additional genes involved in several other behaviors in *C. elegans* (Hodgkin, 1983; Desai, 1988; Thomas, 1990) As many of these mutations affect specific aspects of the nervous system or morphology, observed defects might provide clues as to the roles these affected structures might have in male mating behavior and their nature.

Hodgkin (1983), who developed the protocol upon which the Cod screen is based, also surveyed previously isolated strains for mating ability. In order to provide rigorous measurements in his assay, he tested mating efficiency (ME) alone and did not attempt to characterize any behavioral defects. From the cellular analysis we know that even severe defects in some steps in mating behavior, turning and vulva location, do not necessarily result in the inability to sire cross progeny. So I'm picking up where he left off.

Here, I've used his characterizations, as well as those of subsequent others, as a guide to "prescreen" candidate strains. Specifically, I was looking for the following. First, I was interested in mutants in which specific copulatory structures were affected. General morphological abnormalities were ignored. Second, I looked at mutants with known (or strongly implied) defects in the nervous system. This included defects in sensory organs, neurotransmitter use, and neuronal connectivity. Lastly, I looked at sexually transformed animals, where XX animals are morphologically male.

The tables list the genes chosen for study. In all cases, unless otherwise stated, reference alleles of the genes were used. The second column lists the defect reported in the literature respectively. Where my observations have differed, I note in the footnotes. The third column is the confirmed mating efficiency, where ME3 is wild-type (Nomenclature: Hodgkin, 1983). Lastly, I list any mating defects seen.

Methods

Males occur spontaneously in *C. elegans* at a rate of about 0.2%, not often enough for a graduate student studying male mating behavior. To improve the odds, one of two methods were employed: 1) use of strains carrying the mutation *him-5(e1490)*, which generates 33% males by non-disjunction of the X (Hodgkin et al., 1979); 2) heat shocking L4 hermaphrodites at 30°C for six hours (Sulston and Hodgkin, 1988). This treatment increases the frequency of male progeny from 0.2% to 2-6%. Where possible, a male strain was maintained after heat shock. (Mated hermaphrodites generate 50% males.) However, as I was studying defects in male mating behavior, this wasn't often possible.

Behavioral observations were made as previously described (see chapter 2, Experimental procedures for details). After observation of behavior, the males were mounted for examination under Nomarski microscopy to determine any morphological defects or, if previously reported, verify the reported phenotype. Individual mating efficiency tests were then

set up for each animal. Unless otherwise specified, ten animals of each strain were examined.

Results and Discussion

Copulatory structures

Mutant males in which the rays are missing or abnormal fail to respond, further supporting the evidence from the ablation experiments that the rays are necessary for response (Table 1). Most potentially interesting are the mutants *mab-2* and *unc-62*, with variable numbers of rays. *unc-62* is sluggish but *mab-2* moves well. In *mab-2* animals where at least 6 rays were present, the males were capable of response. Assignment of ray identity was difficult. In general, these findings support those of the ray ablation experiments.

Analysis of mutations which affect the hook and spicules proved uninformative. *lin-1* and *lin-12* males possess additional, ectopic hooks. I examined these mutants to see what possible effects this would have on vulva location. However, males of both strains were defective in response and subsequent examination under Nomarski showed that ray morphology was abnormal.

Sensory organs

The uptake of 5-fluoroscein isothiocyanate by the amphid and phasmid sensillae is an indicator of proper function in those chemosensory organs (Perkins et al., 1986). There is a strong correlation between lack of FITC uptake and lack of response to hermaphrodites (Table 2), suggesting that either the amphid (and/or phasmid) sensillae are necessary for response, or that similar transductional mechanisms mediate both. Three strains are FITC minus yet mate well (Table 2), indicating that the amphids are not necessary for mating. The *osm-3* defect has been shown to be amphid specific. *daf-6* is required in a support (sheath) cell not present in the rays. As the rays are known to be necessary for response, it is likely that these mutants also eliminate function in the rays, suggesting that they are chemosensory.

The *daf-22* phenotype (dauer defective) is suppressible by the addition of exogenous dauer pheromone, suggesting that it is due to a defect in pheromone synthesis or release. Accordingly, I looked at mating between *daf-22* hermaphrodites with wild-type males, as well as the normal mutant male to *unc-31* hermaphrodite pairing. No defects in mating were observed in either case.

Mechanosensory defective males tended to be sluggish, decreasing the frequency of mating encounters. However, when encounters did occur, no defects in mating behavior were observed, implying that mechanosensory transduction is not important in mating.

Neurotransmitters

Mutants deficient in Ach were either lethargic or hyperactive. Due to the general nature of the effect, no mating specific defects could be discerned.

A list of GABA defective mutants was reported in McIntire et al. (1993). All animals tested showed general lethargy and poor backing, but no mating specific defects were observed. Because GABA is the excitatory neurotransmitter used in spicule protraction (E. Jorgensen, pers. comm.), I paid special attention to this step. Spicule insertion was impaired compared to wild type but males were still capable of performing this step. Thus, other neurotransmitter(s) must be involved.

The role of serotonin in turning behavior has been established by Loer and Kenyon (1993). The *cat-2* defect (Table 3) is probably due to defects in the dopaminergic ray neurons (Sulston et al., 1975). The phenotypes of *egl-44* and *egl-45* (Table 3) are reminiscent of the behavior of males in which the PVY interneuron has been ablated, suggesting the possibilities that either PVY is serotonergic or these genes affect some other aspect of PVY function.

It is of note that all 3 known octopamine deficient mutants are also chemosensory defective. *che-3* and *daf-10* are known to have general defects in chemosensory neurons. Thus, the response defect observed is probably due to loss of function of the ray sensillae. *osm-3* is defective specifically in the amphid sensillae, which mediate osmotic avoidance, among other things. Therefore, no role for octopamine in mating behavior is evident.

Connectivity

In *unc-4* mutants, the motor neurons which normally mediate backward motion make connections appropriate for those which mediate forward motion (J. White pers. comm.). The resulting animals, swim forwards well but fail to swim backwards (Brenner, 1974). I observed the mating behavior of these males to determine if the motor system which mediates backing (Chalfie et al., 1985) also mediates backing during mating behavior or if the two were separate. We felt this was a possibility since forward and backward motion are mediated by entirely separable systems (Chalfie et al., 1985). *unc-4* males respond normally to contact with hermaphrodites but fail to back (0/10 males; 0/100 trials), indicating that the motor neurons for backward motion is employed in backing behavior during mating.

Intersex Animals

It has been reported that, while *tra-2* XX animals possess a male body and most of the male nervous system, they fail to respond to contact with hermaphrodites (Hodgkin and Brenner, 1977). I examined *tra-2* animals for mating defects in hopes of dissecting out the key to "maleness". *tra-2* mutants fail to respond (0/10 "males," 0/100 trials); however, examination under Nomarski optics, revealed abnormal ray morphology in these animals. Recently, a new allele of *tra-2* has been isolated, where transformed animals have normal male morphology; these animals mate and sire progeny (Avery,

pers. comm.) as if male. However, given the lineal histories of the parts of the male nervous system, this procedure would be extremely tedious.

Acknowledgments

More so than in any other part of this thesis, this work could not have been done without the services of the CGC (*Caenorhabditis* Genetics stock Center).

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Table 1. Copulatory structures

gene	effects	ME	by male	by trial	Defect
sensory rays					
egl-27	reduced rays/spicules	0	0/10	0/100	response
mab-2	missing rays (6-18)	1	3/10	19/100	response
mab-5	missing rays (all)	0	0/10	0/100	response
lin-22	ectopic rays	2	10/10	100/100	none
lin-28	extra rays	0	0/10	21/100	response
lin-32	rays –> hypodermis	0	0/10	0/100	response
unc-62	missing rays (variable)	0	0/10	8/100	response
vab-10	thin rays	0	0/10	0/100	response
hook					
lin-1	ectopic hooks	0	0/10	3/100	response
lin-12	ectopic hooks	0	0/100	0/100	response
spicules					
egl-2	spicules protrude	1	3/10	8/100	spic. ins
egl-27	reduced rays/spicules	0	0/10	0/100	response
unc-9	spicules protrude	0	0/10	0/100	response

Table 2. Sensory Organs

gene	affects	ME	FITC	Response by male	Response by trial
Chemosensory					
che-1	amphid neurons	2	+/-	10/10	66/100
che-2	ciliated neurons	0	-	0/10	0/100
che-3	ciliated neurons	0	-	0/10	0/100
che-10	ciliated neurons	0	-	0/10	0/100
che-13	ciliated neurons	0	-	0/10	0/100
daf-6	support cells	2	-	10/10	82/100
daf-10	ciliated neurons	0	-	0/10	0/100
daf-19	ciliated neurons	0	-	0/10	0/100
daf-22[a]	pheromone	3	+	10/10	100/100
osm-1	short axonemes	2	-	10/10	67/100
osm-3	amphid neurons	3	-	10/10	96/100
osm-5	short axonemes	1	-	6/10	37/100
Mechanosensory					
mec-3	all mechanorec.	2	+	10/10	83/100
mec-4	"light" touch only	2	+	10/10	88/100

Table 3. Neurotransmitters

gene	defect	ME	by male	by trial	Defect
Serotonin and Dopamine					
cat-1	catecholamine deficient	1	0/10	12/100	turning
cat-2	dopamine deficient	3	0/10	31/100	turning
cat-4	catecholamine deficient	2	0/10	18/100	turning
egl-44	5-HT in some neurons	3	0/10	21/100	backing
egl-45	5-HT in some neurons	3	0/10	15/100	backing
Octopamine					
che-3	octopamine deficient	0	0/10	0/100	response
daf-10	octopamine deficient	0	0/10	0/100	response
osm-3	octopamine deficient	3	10/10	--	none

Chapter 4

Role of the hermaphrodite in *C. elegans* mating behavior: her side of the story

Katharine S. Liu and Paul W. Sternberg

Abstract

Male mating behavior in C. elegans comprises a series of steps: response to the hermaphrodite, turning around her head or tail, vulva location, spicule insertion, and sperm transfer. Previous findings indicate that the behavior is subject to a high degree of external sensory regulation, with each completed step placing the male in the position to receive the next cue. Here we report on our analysis of possible sensory cues provided by the hermaphrodite. Most notably, we show evidence supporting the existence of a hermaphrodite produced signal that is required for successful sperm transfer.

Introduction

At its simplest, behavior is a response to a stimulus - either internal or external. In actuality, most behavior is a constant stream of interconnected stimuli and responses. While each effective stimulus elicits a response, each response will influence the subsequent stimuli the animal will encounter. A perfect example is mating behavior, where in most species, male and female interact, reacting to and providing stimuli from each other. As the "success" of this behavior is highly dependent upon external factors (the partner), a single stimulus, initiating an invariant motor program, would not be sufficient. Instead, sensory cues must be involved in several steps, first to orient the animal to its potential mate, and subsequently, to provide sensory feedback to allow the animal to make required adjustments.

The nature of the stimuli involved has been investigated in a wide range of animals but is probably best characterized in Drosophila. Drosophila courtship behavior involves a complex sequence of steps (reviewed in Hall, 1994). Several sensory modalities have been shown to be involved, including visual, olfactory, and rhythmicity. Narda (1966) first demonstrated that the antennae were needed for successful copulation, indicating that the stimulus was chemosensory. By mosaic analysis, Hotta and Benzer (1976) showed that the signals produced by the male (song frequency) is essential to female response. A female produced pheromone has been identified which is sufficient to trigger courtship behavior directed towards males and inter specifics (Tompkins et al., 1980). A male produced sex-peptide, transferred from male to female, affects subsequent female receptivity (Kubli, 1992)

Mating behavior of the nematode, *C. elegans*, also entails a complex series of steps (Dusenbery, 1980; Liu and Sternberg, 1995). To successfully mate, a male must respond to the hermaphrodite, turn around her head or tail if he encounters them, locate the vulva, insert his spicules, and transfer sperm. However, the situation is not quite so complicated as in *Drosophila*, as this mainly hermaphroditic species does not require mating for propagation. Hermaphrodites do not appear to actively participate in mating and thus are not specifically affected by stimuli from the male (simplifying the problem). Nevertheless, results from ablation studies in the males indicate that sensory signals from the hermaphrodite are required for successful mating (Liu and Sternberg, 1995).

We have investigated the nature and source of these sensory cues using a combination of cellular ablations and genetics. On the starting assumption that the final destination in mating would likely also be the

source of a signal, the hermaphrodite reproductive organs and its associated structures were targeted. Via cellular ablation with a laser microbeam and/or genetic ablation by use of various mutants, we removed potential signal sources in the hermaphrodite and then assayed the ability of intact males to mate with them. In addition, we screened for mutants with hermaphrodite-specific mating defects.

Results

vulva

The hermaphrodite vulva is generated from a pool of six multipotential precursor cells, three of which divide to form vulval tissue (reviewed in Sternberg, 1988). Among the three, the central cell adopts the primary fate, while the remaining two on each side adopt secondary fates. These fates are distinct in cell division patterns and resulting morphology. Thus, the vulva is produced from two distinct cell types.

Ablation of all six precursor cells (or the cell that induces their differentiation) eliminates the vulva. Males respond to and circle these animals normally, eventually swimming away (Table 1). Thus, the cue that elicits response behavior is not produced from the vulva. Ablation of all but one precursor cell (the primary cell) results in a miniaturized, but functional (i.e. - egg-laying competent) vulva. Males locate the vulva, insert their spicules, and transfer sperm with no discernible loss in efficiency (Table 1). Thus, the characteristic shape of the vulva is not required for these steps.

The mutation *lin-12(n137sd)* causes all six vulval precursor cells to adopt the secondary cell fate (Greenwald, 1983), resulting in several ventral protrusions of secondary vulval tissue. Resulting from other effects, this tissue is not connected to the hermaphrodite uterus. Males paired with *lin-12(n137d)* hermaphrodites stop at each ventral protrusion and attempt to insert their spicules (Table 1). Thus, the cue for vulva location is likely produced by the vulval tissue itself, and not secreted from internal reproductive tissues.

Table 1. Manipulations of the vulva

Manipulation	Hermaphrodite Effect		n	vulva location
ablate anchor cell	no induction	no vulva	10	no
ablate all but 1 VPC	1° tissue only	mini vulva	10	yes
lin-12(n137sd)	2° tissue only	ventral bumps	10	yes

In addition to the HSNs (Hermaphrodite Specific Neurons), which have been identified as necessary for egg-laying (Horvitz and Sulston as cited in Trent et al., 1983), the hermaphrodite possesses an additional six sex-specific motor neurons, the VCs, which innervate the vulval muscles. Their function has not been identified, as their ablation has no effect on egg-laying. To test whether these neurons play a part in mating behavior, we removed some or all of these neurons in two ways. First, in mutants carrying a weak allele of the gene *lin-39*, some or all of the VCs die. Hermaphrodites of this genotype were paired with wild-type males to look for possible resulting mating defects (n=20). Second, we ablated all six VCs (n=10) via the precursor cells, P3.aa - P8.aa (Sulston and Horvitz, 1977). This ablation also removes some of the

motorneurons responsible for locomotion (White et al., 1976). In both cases, no discernible defects were found.

gonad

The uterus, associated structures (sheath and spermatheca), and germ line are produced from four precursor cells in the L1 larva (Figure 1B; Kimble and Hirsh, 1979). One of its descendants, the anchor cell, is responsible for induction of vulval cell fate (Kimble, 1981). Ablation of all gonadal precursors but the anchor cell resulted in a hermaphrodite with a morphologically normal vulva that did not connect to a uterus. Males locate the vulva as usual, insert their spicules, but fail to transfer sperm (Figure 1C). Coupled with our previous findings, this suggested to us that a hermaphrodite signal, generated in the gonad and released through the vulva, is required for sperm transfer.

Ablation of the entire gonad is a drastic lesion and the resulting defect in sperm transfer could be due simply to gross morphological distortion of the internal environment. Thus, attempts were made to narrow the site of action. Ablation of the germ line alone (Figure 1C) resulted in no behavioral defects on the part of the males (The hermaphrodites did not lay eggs.) and so the germ line is not necessary for sperm transfer. As ablation of the germ line had no effect, it was eliminated for all subsequent analyses for ease in cell identification. We then ablated subsets of the somatic gonad based upon the blast cells from which they are derived (Figure 1B). In all cases, ablations of subsets of gonadal structures gave intermediate results (Figure 1C). There are three possible explanations. First, the observed defect in

sperm transfer is a non-specific result of the morphological changes. Second, the necessary cue is distributed through out the somatic gonad and its effects additive. Third, the cue is produced by a subset of the gonad but that subset does not correspond to the partitions we have made in our analysis.

Two pieces of evidence favor either of the latter two possibilities. First, ablation of a pair of male sensory neurons called the SPVs results in premature release of sperm outside of the vulva. However, release only occurs in the vicinity of the vulva, suggesting that a signal emanating from the vulva was required to initiate sperm transfer. Second, we have isolated two mutant strains, *sy417*II and *sy418*III, which appear to be defective in the proposed signal (Table 2).

Both strains were isolated in a screen for copulation defective (Cod) mutants in general (chapter 3). Candidate Cod strains were detected by the lack of mating between siblings. In both strains, the reproductive organs appear normal, yet wild-type males fail to sire cross progeny with these hermaphrodites. Meanwhile, their mutant brothers mate with normal efficiencies. The defect is not a result of reduced fertility as brood sizes are comparable to the parent strain. Upon observation, wild-type males locate the vulva, insert their spicules, but fail to transfer sperm (Table 2). This phenocopy of the uterine ablation effect, in two strains which appear morphologically normal, strongly suggests that these mutants lack a signal necessary for the initiation of sperm transfer.

Table 2. Hermaphrodite specific Cods

strain	herm. brood size	male ME	herm. ME
wild type*	210	70%	65%
sy417(II)	165	60%	0
sy418(III)	190	65%	0

**plg-1(e2001d); him-5(e1490)* was used as the parent strain; both mutations are in that background.

Male mating efficiency was measured as described in Hodgkin, 1983.

Hermaphrodite mating efficiency was the number of sired progeny allowed.

Discussion

While behaviorally passive, it is not the case that the *C. elegans* hermaphrodite does not play a role in mating behavior. Her body provides the sensory cues which initiate and coordinate the several steps involved in this intricate behavior. By using the words "cues" and "signals" here, we do not mean to imply information that is actively produced for the purpose of regulating mating behavior. In a species that is predominantly hermaphroditic, it is difficult to speculate about the "purpose" of such cues. Rather, we refer to identifiable components that elicit a response in the male appropriate to mating behavior. These components need not be hermaphrodite specific.

For example, response behavior or the initiation of male mating behavior is not restricted to hermaphrodites. A *C. elegans* male will respond to late stage larval worms, other males, and even itself. Males will also

respond to hermaphrodites (and females) of other species of *Caenorhabditis* (Baird et al., 1992; pers. observations). This lack of specificity is not unique to *Caenorhabditis*. In the genus *Heterodera*, Green and Plumb (1970) reported that most species tested secrete more than one attractant and most males respond to more than one attractant. [In contrast, the differential receptivity between *D. melanogaster* and *D. simulans* is determined by as little as one gene (Coyne et al., 1994; Scott, 1994).] Because of this high degree of redundancy, identification of the eliciting cue(s) for response behavior was not pursued.

A specific cue for vulva location also was not identified. Either primary (in the shape of a hole) or secondary (in the shape of a bump) vulval tissue is sufficient to trigger cessation of backing behavior and the initiation of spicule insertion (or its attempt). From this, we can draw two negative conclusions. The characteristic shape of the vulva is not necessary for its recognition. Any existing signal is produced by the vulval tissue itself and does not depend upon an intact uterus. These results might suggest that any "hole" or "bump" might be sufficient to induce the male to stop. However, *C. elegans* males paired with females of the genus *Amphidirhabditis* do not stop at the vulva, suggesting that they do not recognize it (pers. obs.). Also, *C. elegans* males do not stop at "bumps" of nonvulval tissue, which occur in various mutant strains. Thus, it seems that the cessation of male backing behavior is specifically in response to vulval tissue. That either primary or secondary vulval tissue is sufficient to elicit this response suggests redundancy. In the male, more than one sensory pathway mediates detection of the vulva. Here again, the evidence suggests that multiple, redundant cues are probably acting to guide this crucial step.

Lastly, the combination of male and hermaphrodite ablation results, coupled with the hermaphrodite specific copulation defective strains, strongly suggests that a hermaphrodite signal required for sperm transfer exists and probably resides in the uterine tissue.

Materials & Methods

Strains and Strain Maintenance

The following *C. elegans* strains were used: *him-5(e1490)*(Hodgkin et al., 1979); *plg-1(e2001d); him-5(e1490)*(J. Hodgkin, pers. comm.); *unc-31(e169)*(Brenner, 1974), and *lin-12(n137sd)*(Greenwald, 1983).

Worms were cultured as described by Brenner (1974; Sulston & Hodgkin, 1988) at 20°C, except during behavioral observations which were made at room temperature, around 22-23°C.

Cell Ablations

Since observations for behavioral defects are done with *unc-31* hermaphrodites (see below), the cell ablation experiments were done in that background. The age of the animals selected depended upon the position of the targeted cell along the lineage. The animals were mounted on a glass slide for Nomarski microscopy (Sulston and Horvitz, 1977) on a 5% agar pad with 2-4 mM Na azide (depending on age of animals) as an anesthetic. The targeted cells were ablated by focusing a laser microbeam on the nucleus of

the cell, as described by Sulston and White (1980; Avery & Horvitz, 1987, 1989). Animals were recovered from the slide in M9 buffer and placed onto individual plates with bacterial lawns. A few hours later (about two cell divisions later), the worms are remounted without azide to verify that the proper cell(s) were killed and no unintentional damage was done. Some of the experiments required that cells be lesioned at different times. In these cases, the second round was used to verify the success of the first.

Animals were allowed to mature into young adulthood. They were then observed individually with a group of unadulterated males for resulting behavioral defects in mating. Where appropriate, the males were left on the plate to determine whether they could sire cross-progeny.

Mutant isolation

The two hermaphrodite-specific copulation defective strains described here were isolated in a general screen for copulation defective mutants (Liu, Hajdu-Cronin, and Sternberg, in prep.) Hermaphrodites were examined for their lack of "mating ability" by crossing them with *plg-1(e2001d)*; *him-5(e1490)* males, heterozygous for the marker, *dpy-4(e1166)*. Males heterozygous for *dpy-4(e1166)* normally mate with efficiencies comparable to wild type. The plates were then examined to see if cross-progeny were sired, as evidenced by the segregation of homozygous, Dpy animals.

Observations of Behavior

Observations of mating behavior were done under similar conditions as previously described (Liu and Sternberg, 1995). The behavioral phenotype was determined by observation of intact young adult males with either mutant or operated unc-*31(e169)* hermaphrodites on a 0.5 cm diameter lawn of E. coli (OP50) bacteria. The use of *unc-31* hermaphrodites allowed for observation with hermaphrodites that were sluggish making it easier for the male to keep pace with the hermaphrodite. The criterion for "failure" is the same.

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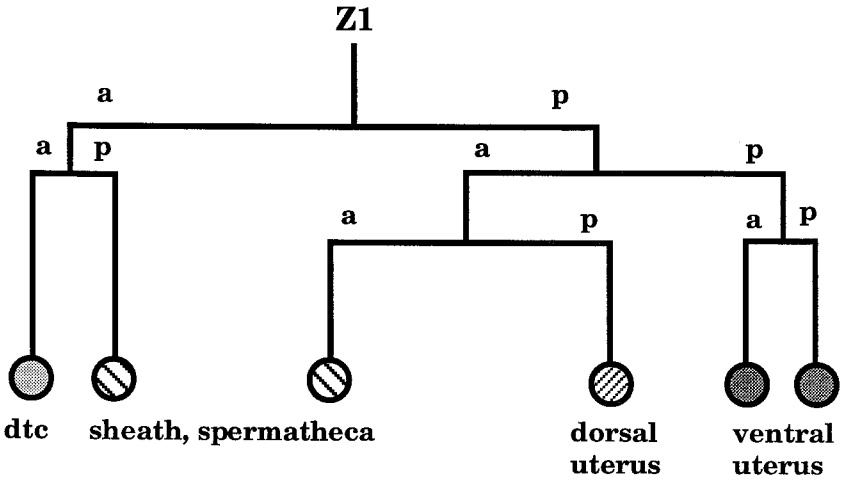
Figure 1. The hermaphrodite gonad

(A) The Z1 lineage: the blast cells Z1 and Z4 produce the somatic gonad while Z2 and Z3 produce germline. Z4 produces the same descendants in a different spatial arrangement. (From Kimble and Hirsh, 1979)

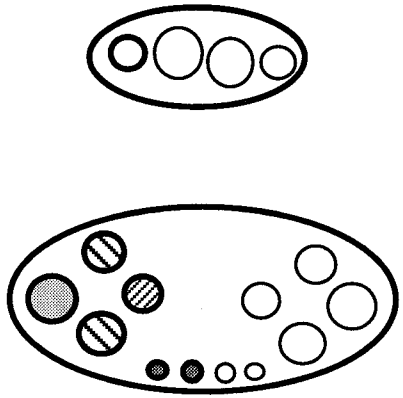
(B) Diagram of the the gonadal precursors in the L1 larval stage and the L3. Textured circles show correspondence with the lineage.

(C) Results of gonadal ablations.

A.



B.



C. Ablation results

Cell(s) Ablated	Missing	Sperm transfer by male	Sperm transfer by trial
none	--	10/10	--
Z2-Z4 / all but Z1.ppp[a]	all but anchor cell	0/6	0/60
Z2-Z3	germ line only	10/10	--
Z1.aa & Z4.pp[b]	distal tip cells	8/8	--
Z1.ap, Z1.paa, Z4.app, Z4.pa[b]	sheath, spermatheca	2/6	2/51
Z1.ap, Z1.pa, Z4.ap, Z4.pa[b]	sheath, sperm., dorsal uterus	2/8	2/76
Z1.pap, Z4.apa[b]	dorsal uterus	1/3	1/28
Z1.ppa, Z4.aaa, Z4.aap[b]	ventral uterus	3/4	3/24

[a] one of the four ventral uterine precursors becomes the anchor cell.
[b] Z2-Z3 were first ablated in the L1 stage.

Chapter 5

Summary

Summary

The actual steps in *C. elegans* mating behavior occur in a stereotyped sequence: response, backing, turning, vulva location, spicule insertion, and sperm transfer. If the male passes the hermaphrodite vulva before he reaches the head or tail, turning behavior is omitted in the sequence. In some strains, a copulatory plug is placed over the vulva after sperm transfer. Males raised in isolation since the first larval stage go through all the same steps of the behavior as normal males, with no observable changes in efficiency. Thus in many respects, male mating behavior looks like a "hard wired" behavior. This reproducibility makes it a good model system for study.

In my introduction, I outlined the current paradigms available to the study of behavior: 1) an ethological approach, entailing direct observation and measurement in order to define behavioral "units;" 2) a cellular approach, using lesions and/or electrophysiology to determine the physical sites that mediate these units; and 3) a genetic approach, involving the identification and characterization of mutations which affect them. In my analysis of male mating behavior, I have used a combination of the three approaches to successfully identify the sensory pathways that mediate each identified step of the behavior and gain insight into the nature of some of these pathways. I have found that the neuronal control of mating behavior in *C. elegans* is not "hard wired" but rather relies on external cues from the hermaphrodite to regulate each step. The reliance on external cues allows the male to adjust his responses in ways appropriate for the perceived environment, including the modification of behavior based on past experience.

This thesis has been broken into parts based on the emphasis in each set of studies. Here I attempt to integrate what we have learned from the different approaches.

response

Two separate lines of evidence suggest that the sensory rays are responsible for the triggering of response behavior. Most directly, ablation of the rays either by ablating the ray tips themselves or by ablating the blast cells from which they are derived, eliminates response of the males to contact (chapter 2). Second, mutants in which the rays are missing or abnormal fail to respond (chapter 3). Ablations of various combinations of the sensory rays suggests that the total number of rays is important for response. Again this is backed up by mutant analysis (Sulston, et al. as cited in Hodgkin, 1983). However, the anterior rays seem to be more important than the posterior, as three pairs of anterior or middle rays is sufficient to mediate response while the three most posterior pairs are not (chapter 2). This may just be due to a positional effect rather than a difference in the properties of the rays.

Several lines of evidence suggest that the rays are chemosensory in nature. By morphology, the neurons look more like chemosensory neurons than mechanosensory (White et al., 1986). Contact with the ray sensory openings is required for response (chapter 2). Most convincing are the mutant studies. Mutants which affect mechanosensation (Chalfie and Sulston, 1981) have no effect on response (chapter 3). The amphid sensillae in the head are known to be chemosensory (Davis et al., 1986). Mutants which eliminate amphid function as evidenced by their ability to pick up

FITC dye (Perkins et al., 1986), also knock out response (chapter 2). There are three known exceptions (Perkins et al., 1986): *osm-1*, *osm-3*, and *daf-6*. The defect in *osm-3* is specific to the amphid ciliary endings. *daf-6* has been shown by mosaic analysis to be in the support cell of the amphids (Herman, 1987), which the ray neurons do not possess (Sulston et al., 1980). Conversely, most of the response defective mutants from our Cod screen are also defective in the amphid function (chapter 3). We currently have one response defective mutant strain in which the amphids dye fill normally (chapter 3). It will be interesting to see what the gene defined by that mutation turns out to be.

While the signal is most likely chemosensory, no mutants have been identified which affect the ability of the hermaphrodite (or other worms) to elicit the response behavior. Hermaphrodites and females of closely related species also elicit the response behavior from C. elegans males and vice versa (Baird et al., 1992 and chapter 4) and the signal is likely highly redundant.

backing

Mating response starts at any point on the hermaphrodite. Thus the exact distance that a male swims before turning cannot be hardwired. But it could be the case that the parameters of the distance within which he will swim are predetermined. In that case, if a male encountered an abnormally long hermaphrodite, he might attempt to initiate a turn prematurely. Premature turns very rarely seen with normal mating behavior. Observation of backing behavior with hermaphrodites of various lengths shows that the males are swimming for many different distances and turning behavior is

always initiated at the appropriate time (appendix 1). Thus, distance is controlled by sensory cues from the hermaphrodite.

Except when mating, the male spends most of his time, like the hermaphrodite, swimming forwards (although he spends more time backwards than she does). Something then must control the suppression of backwards motion and the promotion of forwards motion during mating. This is accomplished by the PVY interneuron, which is located in the ventral cord but has connections into the nerve ring (Sulston et al., 1980). Ablation of this cell in males results in normal response and the initiation of backing but almost immediately, the males swim forward and away from the hermaphrodite (chapter 2). Thus the neuron promotes the continuance of the behavior not the initiation.

Analysis of the behavior of *egl-44* and *egl-45* mutant males gives the same phenotype, although to a lesser extent (chapter 3). These mutants are known to be defective in serotonin expression in several unidentified male neurons in the ventral cord (G. Garriga and B. Horvitz, pers. comm.), raising the possibility that *egl-44* and *egl-45* affect the function of the PVY interneuron and that this interneuron is serotonergic. However, the cat mutants, which are known to be defective in catecholamine synthesis, do not affect backing (chapter 3).

Another question that can be asked about backing behavior during mating is whether it is mediated by the same motor system as normal backing behavior. The neuronal control of movement has been well worked out in *C. elegans*. The motor neurons (White et al., 1976) and command inter neurons (Chalfie, 1985) involved are identified. Surprisingly, forward and backward motions are meditated by completely separate motor systems.

Thus one can eliminate, either by ablation or mutation, one and not the other. Thus, it is not ludicrous to ask whether the backing behavior in mating is separate or not. The mutations *unc-4* and *unc-6* affect the motor neurons that normally mediate backward movement (Brenner, 1974). Observation of these mutant males show that they respond normally but fail to swim backwards (chapter 3) . Thus, the motor neurons used for backing during mating are likely the same used in normal backing. More likely, if there is a difference, it will be in the interneurons which regulate the behavior. The command interneurons which control backward motion, AVA and AVD, have yet to be tested.

turning

We know that the initiation of a turn must depend on some signal from the hermaphrodite. Ablation of the three most posterior sensory rays, generated by the T blast cells (Sulston et al., 1980), results in males which fail to turn, swimming off the end of the hermaphrodite and ending with their tails coiled ventrally (chapter 2). We interpret this behavior as the late initiation of a turn. Two of these rays have neurons which are known to be dopaminergic (Sulston et al., 1975). Ablation of these dopaminergic neurons also results in a turning defect. In this case, males occasionally succeed in turning but do so with a "loose" ventral arch, unlike the characteristic tight ventral coil of intact males (chapter 2). Thus, there appear to be two separable components to a proper turn, the timing and the tightness of the ventral flex (Figure 1). The most posterior T-derived rays seem to modulate when to initiate the turn (ventral flex) and when to end it to continue backing

on the other side. In addition, the rays containing dopaminergic neurons modulate the tightness of the initial flex.

The nature of the signal to initiate a turn is unknown. Strong evidence suggests that chemical cue(s) trigger response behavior, which is also mediated through the rays. However, it is possible for *C. elegans* neurons to be both chemosensory and mechanosensory (Kaplan and Horvitz, 1993). Turning begins at the physical end of the hermaphrodite, just as the degree of curvature increases (chapter 2). The finger-like shape of the rays suggest a mechanosensory mechanism and since the male is swimming backwards during mating, the posterior rays would be in the correct location to detect the end of the hermaphrodite. Of course, this information is also compatible with a chemosensory mode of transduction. Additionally, mutants which disable all known forms of mechanosensation do not affect turning (appendix 3). Thus, if the mechanism is mechanosensory, it must use a different form of transduction than the rest of the worm.

Ablation of the EF interneurons results in a turning defect as well. EF ablated males, swim to the end of the hermaphrodite and stop there, failing to turn (chapter 2). Ablations of both the posterior rays and the EF interneurons results in a defect resembling the EF ablations alone, suggesting that the ray defect is mediated through the EF interneurons (chapter 2).

Loer and Kenyon (1993) have shown by ablation and pharmacology experiments that the CP motor neurons are serotonergic and necessary for normal turning behavior. In their absence, males execute a loose, "sloppy" turn. To a lesser extent, the CA motoneurons also contribute to turning behavior. The ventral coil itself is mediated through the male specific

"diagonal muscles" (Loer and Kenyon, 1993). This is confirmed by one of our copulation defective mutants, *sy35*, which is defective in turning and has been shown to have a sex muscle migration defect (chapter 3). Thus, we have identified sensory, inter, and motor neurons involved in turning behavior. As the phenotype of the CP and sex muscle ablations are similar to the EF ablations, double ablations were not attempted.

vulva location

The hook sensilla detects the vulva as the male is backing and the post cloacal sensillae mediate the adjustment phase as he is attempting to insert his spicules (chapter 2). In the absence of the hook, the post cloacal sensillae, with the spicules, mediate an alternate form of vulva location behavior which is an expansion of the behavior normally only seen in the vicinity of the vulva (chapter 2; appendix 1). Thus, the system is redundant, allowing vulva location via two alternate pathways. There is also plasticity as this alternate form is adopted with subsequent hermaphrodites due to prior experience (appendix 1).

Our initial screen for Cods, which relied on the inability of the males to sire cross progeny with an uncoordinated hermaphrodite, detected no mutants defective in vulva location (chapter 3). This is not surprising given the redundancy and plasticity described above. In our second screen, which relied on the inability of males to mate with their moving siblings, as evidenced by the lack of copulatory plugs over the vulvae, we isolated five mutants defective in vulva location. However, none are as defective as hook ablated animals (chapter 3), implying that affecting this step also causes

other defects that would preclude our isolating such mutants. Interestingly, one mutant exhibits the "slippery vulva" phenotype seen in the ablation of the post cloacal sensillae (chapter 3). Mutant males locate the vulva at frequencies close to wild-type but tend to lose it during the repositioning phase accompanying spicule insertion. This mutant would never have been detected with our first screen.

The neurons that mediate vulva location are most likely chemosensory. The hook structure itself, which looks like a mechanotransducer, is not necessary for proper function of the sensilla (chapter 2). The neurons appear chemosensory by morphology (White et al., 1986) as do those of the post cloacal sensillae. Mutants which knock out chemosensation are of no use because they fail to respond. Attempts to identify a signal via ablation have proved fruitless. Any remaining aspect of the vulva is sufficient to cause a male to stop there (chapter 4). We do know that a functional connection to the gonad is not required, indicating that any signal is inherent to the tissue itself. Also, while *C. elegans* males respond to females of a closely related genus, *Amphidirhabditis*, they fail to recognize the vulva, passing it several times (pers. obs.). As *Amphidirhabditis* must have several differences compared to *C. elegans*, both in chemical and structural composition, we cannot draw any conclusions regarding the nature of the cue. We can say that just having a "hole" in the approximate location is not enough. It could be that the redundancy of male receptors for this step is mirrored by a redundancy of signals in the hermaphrodite. If so, paired ablations of male receptors and parts of the hermaphrodite vulva may prove fruitful.

spicule insertion

Ablation of either the SPD sensory neurons or the SPC motor neurons result in a failure by the male to insert spicules (chapter 2). We propose that the SPDs are required to sense that the male has arrived at the vulva and initiate spicule insertion. Thus, while the hook may signal the male to stop swimming backwards, the signal to initiate spicule insertion is transmitted independently. The actual movement of the spicules would be executed through the SPC motor neurons, which are also thought to be proprioceptive in nature (Sulston et al., 1980). This likely helps with feedback to help insertion. Though normally an inhibitor, GABA has been shown to be the neurotransmitter involved in the SPCs and is excitatory (E. Jorgensen, pers comm). Two sets of muscles are known to be connected to the spicules - the protractors and retractors. Our model proposes that the spicule retractors are constitutively active and that the SPCs are required to inhibit these muscles and excite the protractors during spicule insertion (Figure 2). Alternatively, another set of motor neurons could mediate retraction. However, no obvious candidates exist.

Of all of our Cod mutants, the vast majority affect the step of spicule insertion. Of the nine mutants in this category examined for muscle morphology, only one was found to be abnormal, further suggesting the spicule insertion defects of the other strains are neuronal in basis. Given the number of mutants isolated and the fact that the role of the individual neurons is best characterized in this step, this seems like the best place to begin more detailed (i.e. molecular) analysis.

sperm transfer

While physically associated with the spicules, the SPV sensory neurons actually serve to regulate the transfer of sperm. In their absence via ablation, males either release sperm prematurely or not at all (chapter 2). Double ablations show that the function of the SPVs is dependent upon the function of the SPCs and SPDs. Also, premature release of sperm is never seen away from the vicinity of the vulva, suggesting that a signal from the vulva is necessary to initiate sperm transfer. Thus, sperm transfer is highly regulated, occurring only at the vulva and when the spicules are protracted (figure 2).

Ablation of the DX motor neurons results in constitutive release of sperm from the seminal vesicle into the vas deferens (chapter 2). In normal mating, sperm is not released until spicule insertion occurs. Double ablations with the SPV neurons have the same defect as ablation of the DX's alone. It is difficult to explain how motor neurons which predominantly innervate the body wall muscles would control the release of sperm from the seminal vesicle. It could be that the lesion causes damage or changes the function of other neighboring neurons. Alternatively, the DX's could serve a function independently of their body wall innervation. Other examples of neurons with multiple roles have been demonstrated in *C. elegans* (Sulston et al., 1980; Kaplan and Horvitz, 1993)

Ablation of the hermaphrodite gonad and uterus, leaving the vulva intact, resulted in a failure of males to transfer sperm (chapter 4). Vulva location and spicule insertion are normal. This observation has two implications. First, whatever signal the male uses to locate the vulva and

insert his spicules is contained within the vulva tissue (or shape) itself and not from a substance secreted through it. Second, something about the gonad or uterus is necessary for the initiation of sperm transfer. That something could simply be a physical opening leading to the uterus. Further analysis has shown that the germ line is not necessary for sperm transfer. However, ablations of parts of the uterus and associated structures has given variable results (chapter 4).

That the defect is not due simply to a physical obstruction is greatly bolstered by the isolation of two hermaphrodite-specific copulation defective mutants in our second screen (chapter 3). In both strains, the hermaphrodite reproductive structures look physically normal yet wild-type males fail to transfer sperm, suggesting that they lack a signal necessary for sperm transfer.

While most aspects of mating appear to be controlled by sensory feedback, the amount of time that the spicules are extended into the vulva appears to be internally controlled. In mutant males which fail to transfer sperm (chapter 3), as in mutant and operated hermaphrodites which fail to elicit sperm transfer, the amount of time the spicules are inserted into the vulva is the same as when sperm transfer occurs. Feedback then is not necessary to initiate spicule retraction. This fits well with our model, which requires that spicule retraction be constitutive, with input from the SPC motor neurons required for extension.

Conclusions

The study of behavior as we know it today, started with two general hypotheses. First, it was proposed that behavior consisted of two types - those that were innate and fixed and those which were modifiable by experience (Lorenz and Tinbergen, 1938). It was also believed that while "higher" vertebrates were capable of a wide range of modifiable behaviors, the behavioral repertoire of "simpler" invertebrates was largely limited to that of the hardwired variety. These hypotheses have served as useful models in the undertaking of the study of behavior. However, as in most cases in science, half a century's worth of work has shown that the original distinctions are not so clear cut. Many examples of what were considered to be "fixed action patterns" have, upon further examination, been shown to display plasticity at specific times (Konishi, 1965; Nottebohm et al., 1976). Many examples of behavioral plasticity in invertebrates have been shown (reviewed in Carew and Sahley, 1986).

C. elegans is no exception. *C. elegans* had already been shown to be capable of behavioral plasticity. Both nonassociative (Rankin et al., 1990) and associative learning (Kumar et al., in prep.) have been demonstrated. Stereotyped behaviors are modified by environmental cues. Pharyngeal pumping rates are modified by the availability of food (Avery and Horvitz, 1990) The defecation cycle can be reset by touch (Thomas, 1990). Thus it is not surprising that male mating behavior, which is much more complex than the above behaviors is not entirely hardwired but also subject to modification from sensory input. What might be surprising is the degree to which this is true. Everything that we have learned about mating behavior says that

sensory feedback is involved in every step, and in most cases, multiple pathways mediate each step.

The worm is not so simple as first thought. Within a spartan nervous system is enough information to allow the animal to respond to environmental demands in ways similar to more complex systems, performing sequences of behavior where each step is independently regulated. Thus, the regulation of behavior in *C. elegans* is similar in nature to that of higher animals and serves as an appropriate model system. In addition, despite its ability to modulate its behavior, given constant environmental conditions, its behavior is remarkably stereotyped (reminiscent of it's development), allowing analysis. This combination of complex and modulated behavior but in a simple system, allows us the opportunity to study how steps in complex behaviors are integrated.

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Figure 1. Model of turning based on known morphology (Sulston et al., 1980), data from Loer and Kenyon (1993), and ablation results. Approaching end of hermaphrodite is determined by increase in curvature (loss of contact/resistance), sensed through the posterior sensory rays. Rays signal initiation of turn through EF interneurons; turn is executed primarily through the CP motor neurons exciting the diagonal muscles. Timing and degree of turn are mediated separately by overlapping sets of neurons. Successful turn is signaled by decrease in curvature (regaining contact/resistance).

Figure 1. Model for turning behavior

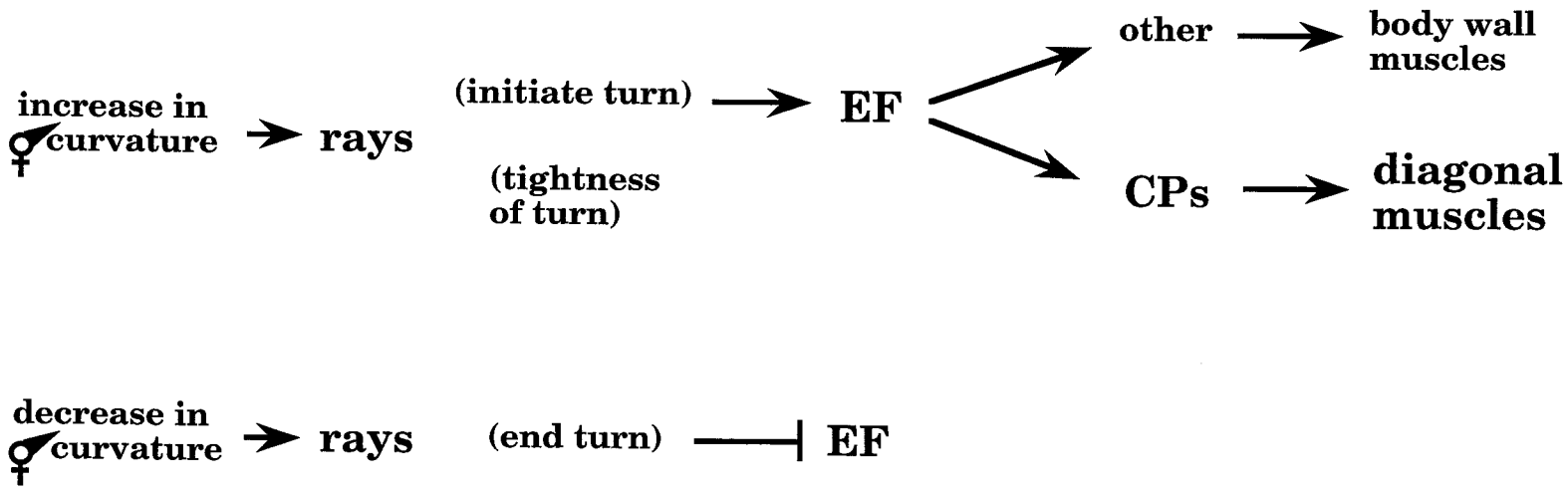

Figure 2. Model of roles spicule neurons play in vulva location, spicule insertion, and sperm transfer, based on known morphology (Sulston et al., 1980) and ablation results. SPD sensory neurons sense arrival at the vulva and signal spicule insertion by exciting SPCs. SPC motor neurons excite spicule protractors and inhibit tonically active spicule retractors, extending the spicules out through the cloaca. A signal in the vulva, together with feedback from SPCs, inhibit SPD sensory neurons, releasing sperm transfer from inhibition.

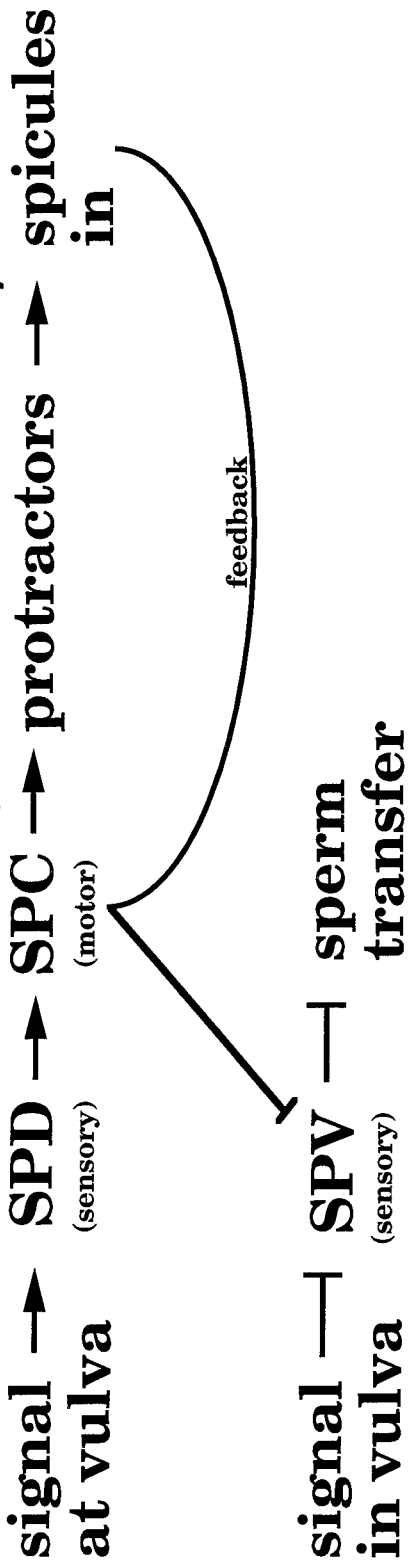

Figure 2. model for spicule insertion and sperm transfer

Appendix

Prior Experience Can Alter Mating Behavior in *C. elegans*

Katharine S. Liu and Paul W. Sternberg

Introduction

One of the steps that a *C. elegans* male must accomplish in order to successfully mate is to locate the hermaphrodite vulva. A structure called the hook is normally employed to accomplish this step (Liu and Sternberg, 1995). Males in which the hook sensilla is intact usually locate the vulva on the first pass. Males in which the hook has been ablated circle the hermaphrodite, passing the vulva several times. However after a latency of about three minutes (200 ± 40 seconds; n=22), these males adopt an alternative backing behavior, slowing their swimming rate and protruding a pair of copulatory structures called the spicules along the length of the hermaphrodite. In intact males, spicule protrusion is seen only in the vicinity of the vulva. By ablation analysis, we determined that a pair of sensory organs called the post cloacal sensillae are required for the expression of this alternate form of vulva location (Liu and Sternberg, 1995).

Neither intact nor altered males adopt this alternate behavior when paired with vulvaless hermaphrodites (Liu and Sternberg, 1995). Therefore, the alternate behavior is not simply the result of neuronal ablation. We argued that the alternate behavior is the expansion of a behavior in the intact male's repertoire. Moreover, expression of the alternate behavior requires the presence of a vulva, suggesting a signal of some sort.

Modification of mating behavior due to experience has been well documented in *Drosophila*. *Drosophila* males attempt to court but soon habituate to mated-females (Siegel and Hall, 1979) and immature males (Gailey et al., 1982). Females previously paired with, but not fertilized by, males are temporarily primed to be more receptive to subsequent males

(Kyriacou and Hall, 1984). Much evidence exists that the chemical cues responsible for eliciting courtship behavior are also used in the modification of that behavior (Tompkins, et al., 1983).

Here, we further investigate what cues are responsible for triggering the behavior. We then address the question of whether this alternate form of behavior constitutes a form of learning.

Results

Further characterization of the alternate behavior

Altered males adopt the alternate behavior after a given amount of time spent in contact with the hermaphrodite and after circling her a number of times. To determine whether interaction time or number of passes over the vulva was the important trigger, we tested the latency to adopting the alternate behavior with hermaphrodites of various lengths. This was accomplished by use of mutant strains that vary body length (see Table 1). Changing the hermaphrodite body length should change the amount of time it takes a male to circle the hermaphrodite and thus change the number of times he passes the vulva in a given amount of time.

Table 1. Varying hermaphrodite body length

hermaphrodite strain	n	relative body length	no. of circles	avg. latency (seconds)
unc-31	22	100%	16.5	200
dpy-13 unc-31	10	60%	23	185
lon-1; unc-31; lon-2	10	165%	7	230

(Relative body length was determined by measuring young adult hermaphrodites with an ocular micrometer. The body length of *lon-1*, and therefore the triple mutant, is variable. For these experiments, the longest animals were chosen.)

Males did circle the different hermaphrodite strains at varying speeds, roughly proportional to the change in body length. Thus, the number of passes made over the vulva varied. However, the latency to adoption of the alternate behavior remained roughly constant. The results suggest that the total time of interaction with the hermaphrodite is the important cue in determining when to switch to the alternate behavior.

Inadvertently, the results further confirm that mating behavior is not simply a series of fixed action patterns, but continually modulated by sensory feedback. The distance spent backing, between executions of turning behavior, is not fixed but varies with the length of the hermaphrodite. This observation further supports our previous ablation results: the initiation of a turn is triggered by sensory cues from the hermaphrodite, mediated through the posterior sensory rays.

Evidence for learning

We wanted to determine if the adoption of the alternate behavior constitutes a similar form of learning. If so, behavior with subsequent hermaphrodites should change as a result of the experience. Males, in which the hook sensilla had been ablated were allowed to grow to sexual maturity in isolation. They were then introduced to hermaphrodites. After the males adopted the alternate behavior, they were then removed for varying amounts of time before being reintroduced with the hermaphrodites (Table 2). The question was, do the males exhibit the alternate behavior immediately or do they start from the beginning as if they were naive?

Table 2. Latencies following "training"

Delay (sec.)	n	Latency (sec.)	Avg. (sec.)
30	5	0; 0; 0; 0; 0	0
60	5	0; 0; 0; 0; 0	0
120	2	0; 7	3.5
300	5	21; 37; 39; 40; 62	40
600	5	66; 147; 189; 234; 314	190

After a brief isolation (30 seconds to two minutes), the animals resume the alternate behavior with no or little delay. This implies that the animals have "learned" and "remembered" that normal vulva location behavior is ineffectual. After a five minute isolation, males again begin with normal vulva location behavior. However, the latency to the alternate behavior is

shortened. After ten minutes the males behave as if they are naive. Thus, the "memory" decays rapidly.

Testing associative learning mutants

By using the terms learning and memory here, we simply mean that the animals behavior has been altered due to prior experience. While superficially the change in behavior looks like a form of sensitization, we cannot guess as to whether the behavior is the result of nonassociative or associative learning. Both have been demonstrated to be involved in Drosophila courtship behavior (nonassociative, Siegel and Hall, 1979 and Gailey et al., 1982; associative, Kyriacou and Hall, 1984). Also, we cannot rule out the possibility that the behavioral change is due to the accumulation of some signal from the hermaphrodite, not requiring any change in neuronal properties.

One way to address these questions is by use of mutants shown to be defective in learning and memory. Failure of these mutants to adopt the alternate behavior would be an indication that the behavior was indeed the result of learning. This method has been used successfully in Drosophila (see above). To test if the observed alternate behavior is the result of associative learning, we obtained two strains, *lrn-1(mm93)* and *lrn-2(mm99)*, which were isolated by their failure to learn under a classical conditioning paradigm (Kumar et al, in press). These mutants have been shown to be specifically deficient in associative learning.

As a control, we first looked at mating ability of these strains as compared to wild-type. *lrn-1(mm93)* and *lrn-2(mm99)* males were tested for

mating efficiency as described by Hodgkin (1983) and were found to be relatively normal (ME3 and ME2 respectively, where ME3 is wild-type). Under direct observation, males of both strains exhibited mating behavior which was not discernibly different from wild type. We then ablated the hook, which mediates normal vulva location behavior and determined whether these males adopted the alternate behavior.

Table 3. Learning mutant latencies

strain	initial	after 30s	after 10s
lrn-1(mm93)	165 (n=6)	180 (n=3)	120 (n=3)
lrn-2(mm99)	170 (n=7)	195 (n=4)	55 (n=3)

[Latencies are given in seconds. As *C. elegans* is mainly hermaphroditic, males for these experiments were obtained by heat-shocking L4 hermaphrodites (Sulston and Hodgkin, 1988). The initial latencies to adopting the alternate behavior were recorded for each male. As the number of available males was low, the pools of *lrn-1* and *lrn-2* males were split in two, with half of the males being reintroduced after 30 seconds. As that time point gave no evidence of learning, males of the second group were reintroduced after only 10 seconds.]

Both strains adopted the alternate behavior with latencies shorter than but not significantly different from wild-type (Table 3: initial latencies). Thus, if this is a form of associative learning, these mutants are not defective in it. However, both strains did vary from wild-type in the amount of time this alternate behavior is retained. While wild-type animals retain this alternate behavior for up to five minutes (Table 2), both learning strains act

as if naive after 30 seconds. *lrn-2* had a decreased latency after a 10 second separation and *lrn-1* appeared not to tolerate a separation.

More data must be collected to draw any firm conclusions, but the evidence suggests that while capable of learning, *lrn-1* and *lrn-2* males are deficient in retention. Several "learning" mutants in *Drosophila* show similar defects (Dudai, 1983). Both *lrn-1* and *lrn-2* have been shown to be defective in both long and short term memory. However, the paradigm used did not allow assay as early as 10 seconds. Even though an associative learning paradigm was used in the screens, most of the learning mutants isolated in Drosophila also exhibit defects in nonassociative learning. The mutants used here are reportedly associative learning specific. A similar characterization of nonassociative learning mutants might help shed further light on the nature of the phenomenon. Unfortunately, mutants for nonassociative learning have not yet been screened for and none are known to exist.

Discussion

As early as 1975, Hedgecock and Russell reported that *C. elegans* was capable of modifying its behavior with experience. Worms placed on a thermal gradient taxed towards the temperature at which they were raised, if food had been plentiful, and away from that temperature, if starved (Hedgecock and Russell, 1975). Recent analyses testing specifically for learning ability have shown that C. elegans is capable of both nonassociative (Rankin et al., 1990) and associative (Kumar et al., in press) forms of learning. *C. elegans* thus joins a large and growing group of model systems for the study of learning and memory (LTP, Bliss and Lomo, 1972; *Aplysia*

Carew and Kandel, 1973; *Hermissenda*, Alkon, 1974; *Drosophila*, Quinn et al., 1974;, Gelperin, 1975)

Mating behavior in *C. elegans* does not require learning. Naive males raised in isolation from larval stages mate upon first introduction to a hermaphrodite and no differences in efficiency can be discerned. However, through prior experience, subsequent mating behavior can be altered. This alteration is not accounted for by maturation, fatigue or injury. By this definition, we claim that males "learn" during their circling of hermaphrodites that the default behavior is not working. They then adopt an alternative behavior to successfully locate the vulva. They keep a short "memory" of this knowledge on the order of about 5 minutes. A rapidly decaying memory is not unprecedented in mating behavior. In *Drosophila* courtship, priming of females to males has been shown to be a form of associative learning and lasts only 1-3 minutes (Kyriacou, 1984).

With this short time period, the effect is obviously not robust. It was not possible, given a single training period paradigm, to establish if the memory would be longer lasting if the training periods were longer. As the males find the vulva soon after they adopt this alternative behavior and do not adopt it if with a vulvaless hermaphrodite, the "training" period is necessarily only the latency to adopting the alternative behavior. Plasticity provides more evidence that the motor program for mating behavior, while innate, is not a "fixed" action pattern.

We are assaying a behavior exhibited by surgically altered animals. Is it at all relevant? We have previously shown that the alternate behavior is not a novel behavior brought on by ablation and argued that it is an expansion of a behavior that is normally seen in the vicinity of the vulva. An

intact vulva is necessary for the expression of this alternate behavior, suggesting that a diffusable signal, originating from the vulva, is detected as male circles hermaphrodite. Analogous to the studies done in *Drosophila*, this behavior may result from an association between the detection of a vulval signal and the act of backing.

If the purpose of the ability to alter behavior due to prior experience is to increase the fitness of the animal, whether to locate or avoid, then the ability to modify mating behavior would certainly fit in.

Acknowledgments

I thank Jeff Hall for suggesting that we try assaying known learning defective mutants and Derek van der Kooy for generously sending me his brand new mutant strains.

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