

FUNCTIONAL DISSECTION OF THE SENSORY RAYS IN
CAENORHABDITIS ELEGANS MALE MATING BEHAVIOR

A Thesis

by

PAMELA KRISTINE KOO

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

MASTER OF SCIENCE

December 2010

Major Subject: Genetics

Functional Dissection of the Sensory Rays in *Caenorhabditis elegans* Male Mating

Behavior

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Approved by:

Chair of Committee,	Robyn Lints
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ABSTRACT

Functional Dissection of the Sensory Rays in *Caenorhabditis elegans* Male Mating Behavior. (December 2010)

Pamela Kristine Koo, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Robyn Lints

The nematode *Caenorhabditis elegans*, with its sequenced genome, compact nervous system and stereotyped behaviors is an ideal model organism in which to study the integration of sensory input with motor output. Male mating behavior is among the most complex of these behaviors and males utilize a number of sensory organs in its execution. Among these are the rays, which are nine pairs of sensory organs that are arranged laterally along the male tail. Each ray is composed of two ultra-structurally distinct neuron types, an A type and a B type, surrounded by a glia-like structural cell. Though compositionally identical, each pair of rays maintains a unique, genetically-hardwired identity based on wiring, morphology, and neurotransmitter fate. Three techniques were used to investigate the role of the rays in male mating behavior. First, cauterization of the tips of the rays removed their sensory endings, leading to ray neuron death. Second, a heterologous light-activated cation channel was utilized to activate specific ray neuron types. Finally, ray neuron types were genetically targeted to undergo apoptosis by expression of heterologous caspases.

The results show that the rays play important roles in multiple steps of male mating behavior, including contact response, scanning, and turning. The rays as a whole mediate posture change and backing during contact response. The ability to respond to hermaphrodite contact is shared among the rays, as is initiation of backward locomotion, though all rays are required for efficient, prolonged backward scanning. Both A and B neuron types appear capable of initiating contact response. Direct activation of B neurons through ChR2 causes a contact response-like ventral tail flexure, and elimination of both A and B neurons reduces contact response. A neurons additionally have a unique role in turning.

DEDICATION

To Andrew J. Gray, in thanks for friendship and support

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CHAPTER I

INTRODUCTION

Organisms must react appropriately to changes in their surroundings in order to survive. The mechanisms by which organisms sense their environment and translate such information into a suitable response is thus a basic question of science. Such mechanisms occur on many levels – systemic, cellular, and molecular. Obtaining answers to these questions is often made difficult, if not impossible, by the complexity of living organisms. The nematode worm *Caenorhabditis elegans*, however, is an ideal model organism in which to study the integration of sensory information and motor output. It possesses a simple and well-characterized nervous system, is genetically tractable, and performs a variety of behaviors.

C. elegans is a soil nematode approximately 1 mm in length. Hermaphrodites have just under 1000 somatic cells and exactly 302 neurons, while males have 378 neurons [1]. The identity and developmental lineage of every cell is known [2-4]. The neuronal circuitry of the hermaphrodite has been mapped, and the male is currently in reconstruction with the posterior portion complete [1, 5]. This allows the unprecedented opportunity to generate hypothetical circuitry maps with single cell resolution.

The *C. elegans* genome has been sequenced, and a number of genetic tools are available including a wide variety of mutants, tools for forward and reverse genetics, and

This thesis follows the style of Current Biology.

the ability to readily produce transgenic animals in the lab [6-10]. As the *C. elegans* body is transparent, it is a relatively simple process to observe expression patterns for any number of genes by driving fluorescent protein expression with the promoter for the gene of interest [11]. In a similar fashion, targeted expression can be used to rescue mutations or manipulate cell function through the use of heterologous proteins.

An assortment of behaviors has been examined in *C. elegans* at both the neuronal and molecular level including chemotaxis, thermotaxis, and social behavior [12-15].

The large majority of behavior, however, is studied in hermaphrodites. *C. elegans* is an

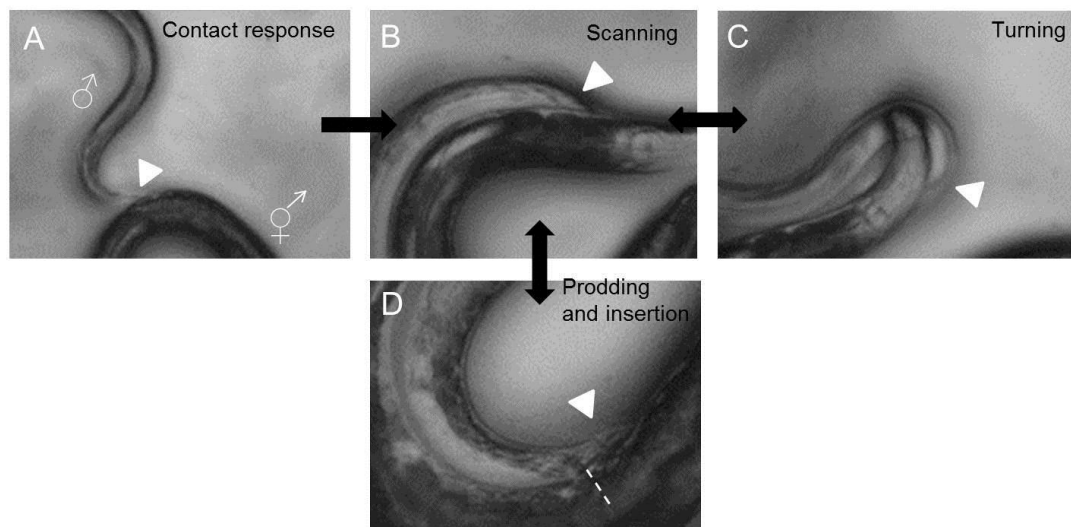


Figure 1.1. Male Mating Behavior Consists of Multiple Sub-behaviors

Male mating behavior is comprised of a series of sub-behaviors. The white triangle indicates the male tail throughout. A. Contact response. When the male tail comes in contact with the hermaphrodite, he ceases forward locomotion and alters his posture to press his tail ventrally along the hermaphrodite cuticle. B. Scanning. The male proceeds to back along the hermaphrodite cuticle in search of the vulva. C. Turning. If he reaches the head or tail end of his mate, the male performs a sharp ventral turn to reach the opposite side of the hermaphrodite, then resumes scanning. D. Prodding and insertion. When the male locates the vulva (dashed white line), he ceases backwards locomotion and prods rapidly against the vulva opening to part the vulva walls. Upon doing so, he extends his spicules completely and inseminates his mate. Males move interchangeably between scanning, turning, and prodding as necessary until insemination occurs.

androdioecious species which produces males at a low frequency. Hermaphrodites are self-fertilizing and their role in mating behavior is passive at best, and ordinarily they are reluctant partners [3].

Male mating behavior is arguably the most complex of the *C. elegans* repertoire and consists of a series of sub-behaviors (Fig 1.1) [16, 17]. When the male tail contacts the hermaphrodite, the male ceases forward locomotion, alters tail posture with a ventral flexure that presses his tail against the hermaphrodite cuticle, and initiates backwards locomotion along the hermaphrodite in search of the vulva. This entire sequence is known as contact response (Fig 1.1A). Backwards locomotion along the hermaphrodite cuticle, a sub-behavior called scanning, continues until the male locates the vulva or reaches the head or tail end of the hermaphrodite (Fig 1.1B). If the male reaches either end of the hermaphrodite he performs a sharp ventral turn that allows him to back along the opposite side (Fig 1.1C). When the male locates the vulva, he ceases backwards locomotion and prods rapidly with his spicules in an attempt to part the vulva walls. When he succeeds, he inserts his spicules fully into the vulva and inseminates his mate (Fig 1.1D) [16, 17]. The male is able to switch between scanning, prodding and turning as necessary until insemination occurs. A male can, for instance, initiate prodding without having turned on a particular hermaphrodite.

Males utilize a number of muscles, support cells, and neurons in the attempt to copulate with his partner. Various steps in male mating behavior have been explored at both the cellular and molecular level. Ablation studies implicate the sensory rays in

contact response, scanning and turning (Fig 1.2A) [17]. Contact response can be disrupted by loss of TRPP homologs or α -tubulins in the ray neurons [18-20].

Downstream of the rays, turning posture requires a number of sex-specific muscles. Elimination of these muscles leads to males who make wide instead of tight ventral turns, and ablation of the serotonergic CP interneurons which innervate these muscles results in a similar though milder phenotype [16]. Complementary to these experiments, application of exogenous serotonin (5-HT) to males causes tight ventral curling of the tail which becomes looser when the sex muscles are ablated [16]. This suggests both that the sex-specific muscles are involved in the tail posture required for turning and that muscles common to both sexes, such as the body wall muscles, contribute to a male-specific behavior. The dorsal body wall muscles have been shown to control dorsal curling stimulated by acetylcholine (ACh) [21]. This dorsal curl is hypothesized to play a role in balancing the contraction of ventral muscles during backwards scanning [21].

Vulva location is by far the best understood step of male mating behavior. This step is mediated through a pair of neurons in the hook sensillum, HOA and HOB, and three pairs of neurons in the post-cloacal sensillum (PCS) - PCA, PBC, and PCC (Fig 1.2A) [4, 17, 22]. The spicules are controlled by two pairs of muscles (a pair of protractors and a pair of retractors) and several neurons [4]. Spicule activity at the vulva consists of two stages – initial fast contraction of the protractor muscles to cause high frequency prodding and sustained contraction of the protractor muscles once the spicules have penetrated the vulva lips [22]. Contraction of the protractor muscles is stimulated

by ACh released from the PCB and PCC neurons of the post-cloacal sensilla, and the spicule neuron SPC. Rapid prodding is mediated by PCB and PCC signaling, while prolonged contraction of the protractors results specifically from the activity of SPC [22]. Acetylcholine promotes spicule contraction through multiple acetylcholine receptors including the nicotinic acetylcholine receptors (nAChRs) UNC-38 and UNC-29 and ACh activity is potentiated by the muscarinic acetylcholine receptor GAR-3 [22, 23]. Potassium channels like the *ether-a-go-go* like potassium channel UNC-103 serve to modulate circuit excitability to prevent premature spicule protraction [24].

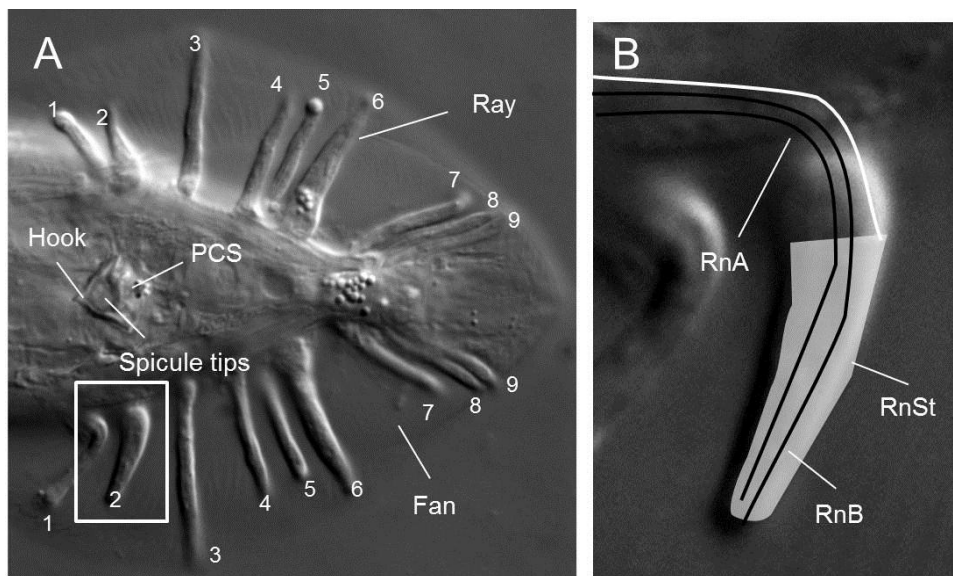


Figure 1.2. Sensory Organs of the Ventral Surface of the Male Tail and Structure of the Rays

A. The ventral surface of the male tail contains a number of sensory organs. These include the hook, the post-cloacal sensilla (PCS), the spicules and the rays. Rays are conventionally numbered 1-9 anterior to posterior. The rays extend out from the body and are embedded in an acellular fan. B. Diagram of structure of rays, enlarged from box in A. The rays contain the dendritic endings of three cells, an A type neuron (RnA), a B type neuron (RnB) and a glia-like structural cell (RnSt) which surrounds the two neuron types. A neurons remain within the ray structure and contain a striated rootlet. B neurons have a ciliated tip which extends out into the environment through an opening at the ray tip.

While the precise mechanisms by which sperm transfer occurs is unknown, some of the molecular underpinnings have been explored. IP3 signaling, likely in the somatic gonad of males, and vesicle exocytosis in both the ventral cord motor neurons and the gonad is required for efficient sperm transfer [25, 26]. Timing of sperm release is controlled by the SPV sensory spicule neuron and the CA neurons in the ventral nerve cord appear necessary for the initiation of sperm transfer [17, 26].

This work focuses on the function of the sensory rays in male mating behavior. The rays consist of nine pairs of finger-like projections located on the lateral surface of the tail, conventionally numbered 1-9 anterior to posterior. The rays extend out from the tail into the environment and are embedded in an acellular fan (Fig 1.2A) [4]. The rays are compositionally identical. Each contains the dendritic ends of two ultra-structurally distinct neuron types and a glia-like structural cell, all three surrounded by a layer of hypodermis and cuticle (Fig 1.2B). Each ray is open to the environment at their tip, with the exception of ray 6. The A type neuron remains within the hypodermal structure and is marked by a striated tip. B type neurons end in a primary cilia which extends into the environment in all but ray 6 (Fig 1.2B) [4]. Despite being compositionally identical, each pair of rays possesses their own unique identity defined by the pattern of neurotransmitters synthesized in the A and B neurons, morphology of the ray, and direction of the ray opening [2, 4, 16, 27, 28].

The rays make numerous electrical and chemical synapses – between A and B neurons within a ray, within pairs, between pairs of rays, and with other neurons, interneurons, and muscles [5]. While many of the targets of ray neurons are similar,

each pair of rays also appears to have some unique identity in the specific numbers and types of connections made. Access to recent work reconstructing the wiring of the male nervous system allows for hypotheses on the role of rays in male mating behavior [5].

In the current work, we show the rays are the primary sensory organs that mediate contact response, a sub-behavior that can be further divided into two parts – backing and posture change. All subsets of rays appear capable of initiating contact response, but function additively in scanning such that an increased number of rays prolongs any single episode of scanning. Contact response can occur in the absence of all neurotransmitters tested, and can occur in the absence of ray neurons that make direct muscle connections. A-neurons mediate multiple steps of mating behavior including contact response, scanning, and turning. B neurons play a more secondary role, possibly functioning to facilitate A neuron driven behavior.

CHAPTER II

MATERIALS AND METHODS

Strains

For these experiments, the following strains were used – *mnIs17[osm-6::gfp + unc-36(+)]* (a gift from C. Spike and R. Herman), *nIs128[ppkd-2::gfp]* (H. Schwartz and H.R. Horvitz), *bxIs19 [ptrp-4::gfp + ttx-3::gfp]*; *bxIs20 [ptrp-4::caspase3-NZ + pgrd-13::CZ-caspase3 + elt-2::gfp]* [29], *fsIs14 [ptba-9::yfp + ptba-6::mCh + psulp-3::gfp]* (a gift from R. Miller and D. Portman), *unc-64(e246)III*, *ceh-30 (n4111 n3714)X* [30]. All strains were in the *him-5(e1409)* background [31]. All strains were cultured under standard conditions [32] and maintained at 20°C.

Plasmid construction

To construct the plasmids *ppkd-2::ICE* and *ppkd-2::Chr2-yfp*, the Gateway vector system was utilized (Invitrogen). The 1.7kb region upstream of the PKD-2 ORF was amplified from genomic DNA with primers containing additional attB sites. (primers are *ppkd-2*: ATTB1pkd2: ggggacaagtttgtaaaaaagcaggctgcaaccagcagttgtaaattcgg and ATTB2pkd-2:ggggaccactttgtacaagaaagctgggtgaagacggctcgcgtgaaacagtag or *ppkd-2rev*: ATTB2pkd-2:ggggaccactttgtacaagaaagctgggtgcaaccagcagttgtaaattcgg and ATTB1pkd-2:ggggacaagtttgtaaaaaagcaggcttgaagacggctcgcgtgaaacagtag) This PCR product was combined with the pDONR221 vector in a BP reaction to create the *ppkd-2* entry vector *ppkd-2* ENTRrev (pZL10) or *ppkd-2* ENTR (pZL1). These promoters were then cloned to the appropriate destination vector, either *ccdBrev::ICE* (pZL16) or *ccdB::Chr2-yfp* (pZL5 or pLR167) in an LR reaction.

The *ccdBrev::ICE* destination vector was created by excising the *pdat-1* promoter from the *pdat-1::ICE* construct (Hills et al 2004) with *SmaI* and *PstI*, end filling, and then inserting the *ccdB* cassette C.1 (Invitrogen) in the reverse orientation. *ccdB::ChR2-yfp* (pZL5) was created by digesting *mec-4::ChR2(H139)-yfp* (a gift from Alexander Gottschalk) with *HindIII* and *BamHI* to excise the *mec-4* promoter, end-filling, and inserting the *ccdB* c.1 cassette. This insertion created a stop codon (TGA) at the cassette/vector junction which was changed to a glycine codon (GGA) by site-directed mutagenesis to generate pLR167.

The plasmid *pgrd-13* (pZL17) contains only the *grd-13* promoter and was used to generate control strains (*fkEx36*, *37*) for A neuron caspase experiments. It was derived from EM#317 [29] by digesting with *EcoRI* and *BamHI* to remove the *CZ-caspase3* sequence, end-filling, and then re-ligating the cut plasmid.

Transgenic strains

Transgenic lines were generated by Robyn Lints using standard microinjection techniques [6]. The following strains were used in this study.

fkEx9 [*ppkd-2::ChR2-yfp* + *punc-122::gfp*]

fkEx12: [*ppkd-2::ICE* + *punc-122::gfp*]

fkEx13: [*ppkd-2::ICE* + *punc-122::gfp*]

fkEx30: [*punc-122::gfp*]

fkEx36: [*ptrp-4* + *pgrd-13* + *elt-2::gfp*]

fkEx37: [*ptrp-4* + *pgrd-13* + *elt-2::gfp*]

The above arrays were generated by injecting plasmids at the following concentrations: *ppkd-2::ChR2-yfp* (20ng/ μ L); *ppkd-2::ICE* (70ng/ μ L); *ptrp-4* (25ng/ μ L); *pgrd-13* (25ng/ μ L); *elt-2::gfp* (50ng/ μ L); *unc-122::gfp* (50ng/ μ L). The total DNA concentration of the injection mixture was made up to 150-200ng by supplementing with pUC18 plasmid DNA.

Mating behavior assays

To make assay plates for each set of trials, two aliquots of 1 mL OP50 from an overnight culture were centrifuged at 6000 rpm for 5 minutes, the supernatant was reduced to 50 μ L per aliquot, and the bacterial pellets were resuspended and combined to yield 100 μ L concentrated OP50 solution. 5 μ L of this concentrated solution was spotted onto individual plates and allowed to soak in, providing fresh bacterial lawns approximately 5 mm in diameter. All plates were seeded the day of the trials to be performed.

For each trial, five *unc-64(e246)* hermaphrodites were placed on a bacterial lawn and given at least 10 minutes to acclimate and cease movement. A single male was placed on the lawn, and a timer started. The male was allowed 15 minutes to attempt to mate, and selected male behaviors were recorded by an Excel macro which logs keystrokes and timestamps [23]. Assays were performed on a Zeiss Discovery stereomicroscope and were digitally recorded using a Zeiss AxioCam HS and AxioVision software. Trials ended at the conclusion of 15 minutes or after the male ejaculated, whichever occurred first.

Percent contact response: When the male tail comes in ventral contact with a hermaphrodite, he changes tail posture with a ventral flexure of his tail, ceases forward locomotion, and begins to move backward, pressing his tail ventrally into the hermaphrodite cuticle. Males who are deficient in contact response can respond in several ways: ventral flexure of tail with no backing, backing with no subsequent posture change, or no response at all. During the trial, each individual tail contact was scored in one of four categories: normal contact response, backing without posture change, posture change with no backing, and no response. The percent of normal contact responses initiated was calculated by dividing the number of normal contact responses by the total number of contacts made (Eq. 1).

$$Eq. 1 \quad \% \text{ contact response} = \frac{\text{No. of contact responses}}{\text{Total no. of tail contacts}}$$

Average duration of tail contact: To calculate the average duration of tail contact, the total time that the male was involved in a step of mating (contact response, scanning, turning, prodding at the vulva or ejaculation) was calculated from the Excel spreadsheet and was divided by the total number of contacts made during the trial (Eq. 2).

$$Eq. 2 \quad \text{Average duration of tail contact} = \frac{\text{Time spent with tail in contact}}{\text{Total no. of tail contacts}}$$

Laser cauterizations and ablations

Two strains were used for these experiments: *mnIs17[osm-6::*gfp* + *unc-36*(+)]* which expresses in most ciliated neurons including the A and B type ray neurons (Collet et al 1998) and *fkEx9[ppkd-2::*ChR2-yfp*]* which drives expression of *ChR2-yfp* in the B-

type neurons of rays, the HOB neuron of the hook sensillum, and the male specific CEM head neurons [18]. To cauterize the rays, males were picked as virgin L4s (5 males per plate) and isolated from stock plates. Approximately twenty hours later, individual adult males were mounted on 5% agarose pads with 2 mM NaN₃ as an anesthetic. The tips of the rays were cauterized with a laser until cytoplasm from the ruptured cells flowed freely. To control for the effects of anesthetic in either strain, males isolated in the same fashion were exposed in an identical manner to 2 mM NaN₃, but were not laser-operated. In both strains, all males were allowed to recover from anesthetic effects for at least five hours before being assayed. After trials were performed, cauterized males were examined under a compound microscope equipped with fluorescence to examine ray integrity and GFP or YFP expression.

The CEMs were laser ablated by standard procedures [33]. *nIs128[ppkd-2::gfp]* males were isolated as juvenile L4s (10 to a plate). Males were placed on 5% agarose pads containing 2 mM NaN₃ as anesthetic with control males similarly treated but not subjected to laser surgery. GFP expression in the CEMs was used to identify the cells for laser ablation in young adult males (< 5 hours post-final molt). Males were tested approximately 20 hours post- ablation in standard mating assays. Death of the CEMs was confirmed by the absence of GFP expression under a compound microscope equipped with fluorescence.

Channelrhodopsin-2 (ChR2) assays

To specifically activate the B neurons, the light-inducible cation channel ChR2 from *Chlamydomonas reinhardtii* under the control of the promoter for the TRPP homolog PKD-2 was utilized [18, 34]. *fkEx9[ppkd-2::ChR2-yfp + punc-122::gfp]* animals used for ChR2 assays were maintained on plates seeded with OP50 *E. coli* containing 50 μ M all-*trans* retinal (ATR) [34]. For assays, L4 virgin males were isolated from stock plates (5 males per plate) on plates containing ATR, freshly spread and allowed to dry the day of isolation. As ATR is light-sensitive, plates were kept wrapped in foil except when trials were being performed or plates were being maintained.

ChR2 assays were conducted approximately 24 hours after isolation and were performed on a stereomicroscope equipped with fluorescence. Trials were recorded using a Zeiss AxioCam HS and AxioVision software. For each male tested, the assayer waited until the male was moving forwards on the plate before opening the fluorescence shutter for a period of approximately 500 ms.

Males undergoing laser surgery to cauterize rays and/or ablate HOB were operated on according to previously described protocols and allowed to recover for at least five hours prior to testing. If mating assays were done after ChR2 assays, males were moved from the assay plate to a plate seeded with OP50 and allowed at least 15 minutes to recover before the mating assay was performed.

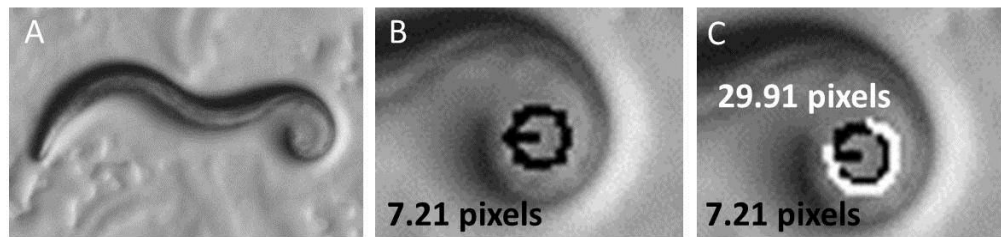


Figure 2.1. Measurement of ChR2 Tail Curl

A. To measure ChR2 induced tail curl, the frame showing the maximum tail curl after a blue light pulse was extracted from videos. B. A circle (in black) measuring the radius (in pixels) of the tail curl was drawn using the tools available in Zeiss' AxioVision software. C. To account for the tightness of the tail curl, the amount of the male's tail in direct contact with the previously drawn circle was traced (in white) using the curve spline tool available in Zeiss' AxioVision software. This value was used to generate a correction factor by dividing the circumference of that circle ($2 \times \text{radius} \times \pi$) by the amount of tail in contact with the circle. The final ChR2 tail curl value was determined by multiplying the circle radius by this correction value.

To quantify the tail curl response in the ChR2 assays, a circle was drawn whose circumference ran along the inside curve of the tail using the circle measurement tool in the Zeiss AxioVision software. The radius (in pixels) of this circle is proportional to the curvature of the tail curl. This value was multiplied by a penalty factor, calculated by dividing the circumference of that circle by the amount of the tail in contact with the drawn circle such that males who form a closed circle with their tails received a ratio of 1 and males with more open tail curls received a penalty which increased the tail curl measurement (Fig. 2-1, Eq. 3).

$$\text{Eq. 3} \quad \text{ChR2 tail curl} = \text{tail curl radius} \times \frac{\text{circumference of tail curl}}{\text{tail contact with circle}}$$

Screening of A and B neuron ablated animals for mating assays

B neurons were eliminated through genetically-targeted expression of ICE through the PKD-2 promoter [18, 35]. The genotype for these strains was *fkEx12* and

fkEx13[ppkd-2::ICE + unc-122::gfp]; nIs128[ppkd-2::gfp]. ICE expression in these lines led to death of the B neurons, the HOB neuron of the hook sensillum, and the male-specific CEMs in the head [18]. Virgin L4 males were separated from the stock population (20 males to a plate) and allowed to mature overnight. Males with no tail fluorescence were isolated on a stereomicroscope equipped with fluorescence an hour prior to mating assays and their mating behavior subsequently observed. Absence of B neurons was later confirmed by examination of GFP fluorescence under a compound microscope equipped with fluorescence. *fkEx30[punc-122::gfp]; nIs128* was used as a control for these experiments.

A neuron reduction was accomplished by neuron-specific expression of reconstituted human caspase-3 [29, 36]. The genotype of the strains used for these experiments was *bxIs19[ptrp-4::gfp]; bxIs20[ptrp-4::caspase3-NZ + pgrd13::CZ-caspase-3 + elt-2::gfp]* [29]. *elt-2::gfp* positive animals were isolated as virgin L4 males and separated from the stock population (5 males per plate). The extent of RnA-cell death was determined after mating assays by examining *ptrp-4::gfp* expression under a compound scope equipped with fluorescence. Due to mosaic expression of the *trp-4* promoter, cell death was only reliably observed in three A neurons – R3A (absent in 90% of males), R5A(90%), and R8A(85%). More variable levels of cell death were observed in R2A(25%) and R6A(25%). *fkEx36* and *fkEx37; bxIs19[ptrp4 + pgrd-13 + elt-2::gfp]* were used as controls for these experiments.

CHAPTER III

FUNCTION OF THE RAYS

Introduction

Previous studies implicated the rays in the first step of male mating behavior, contact response [17]. To determine their role in mating behavior, Liu and Sternberg eliminated various combinations of rays by ablation of the ray precursors through laser surgery and observed a high degree of redundancy amongst rays in the initiation of contact response. However, as all combinations of ablations in their experiments left either some rays or other ventral sensory neurons in the tail intact, the role of rays themselves apart from other ventral sensilla remained unknown.

Ray neurons make chemical and electrical connections amongst themselves, between A and B type neurons within a pair as well as between various pairs. In addition, ray neurons wire to numerous interneurons, motor neurons, and directly to muscles as well as making connections to other sensory neurons [5]. The wiring thus suggests that the rays alone are sufficient to initiate contact response. However, as other sensory neurons in the tail also make many of the same synaptic connections as the rays, especially to common downstream interneurons, the rays might not be necessary for contact response [5].

To determine the role of the rays apart from other sensory neurons, mating behavior was examined in males with all rays disabled. Males lacking rays were unable to make the proper ventral flexure posture change upon ventral tail contact with the

hermaphrodite cuticle and backed upon contact at a highly reduced rate. This suggests that rays are the primary mediators of contact response, which can be separated into two sub-steps – backing and posture change.

Results

To investigate the role of rays as a whole in contact response, the tips of each ray, which includes the ends of the sensory dendrites, were rendered nonfunctional with a laser. To ensure that the dendritic tips were eliminated, ablations were initially done in *osm-6::gfp* males which express GFP in ciliated sensory neurons including the A and B type neurons of the rays, accumulating in the ciliated tips of these neurons [18, 37]. Visible damage to the ends of the rays in addition to the absence of GFP expression at the tips of the ray neurons suggest that the dendrites have entirely lost their ciliated ends. Such cells are not expected to sense stimuli, as males with shortened or stunted, as opposed to absent, cilia perform poorly at behaviors such as vulva location and attraction to hermaphrodite pheromones [18, 38]. Additionally, calcium increase in response to normal stimuli is eliminated in ciliated neurons completely lacking cilia, suggesting these cells no longer respond to stimuli [39]. Absence of GFP in both the dendritic ends of the ray neurons and the cell bodies in operated males suggests that both A and B neurons in their entirety were killed in the cauterizing process. Rays in which GFP was

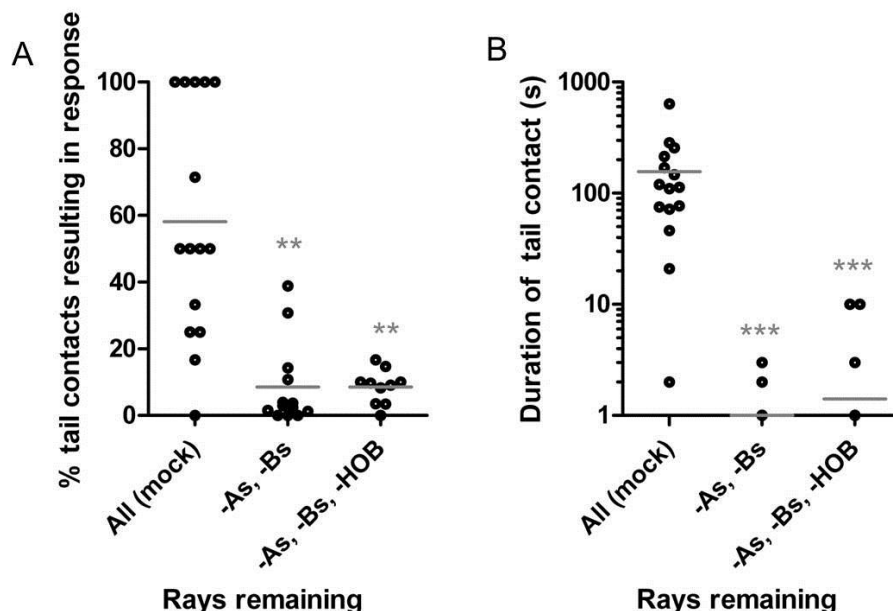


Figure 3.1. The Rays Are Required for Contact Response and Scanning

A. Loss of the sensory rays significantly reduces males' ability to respond to contact. Additional loss of the HOB sensory neuron of the hook does not further reduce contact response. Animals were subjected to laser surgery to eliminate the sensory endings of the ray neurons. Mock animals were similarly exposed to anesthetic but not subjected to surgery. For each animal, the percent of tail contacts which resulted in contact response was calculated by dividing the number of normal contact responses by the total number of tail contacts that occurred in the trial. Each circle represents one male, gray lines indicate means. ** = p-value < 0.01 as calculated by nonparametric ANOVA, compared to mock ablated animals. B. Loss of the sensory rays significantly diminishes the amount of time a male spends in tail contact with his mate. Duration of tail contact was calculated by dividing the total amount of time the male spent with his tail in contact with the hermaphrodite cuticle by the total number of contacts made by the male during the trial. Each circle represents one male, gray lines indicate means. *** = p-value < 0.001 nonparametric ANOVA, compared to mock ablated animals.

bleached were GFP-positive when examined five hours later, implying that the absence of GFP expression is not due to bleaching during the ablation process (data not shown).

Males lacking all rays respond to contact at a significantly reduced rate (Fig. 3.1A). In intact males, tail contact with a hermaphrodite ordinarily induces posture change and backing along her cuticle from. In contrast, males with all rays cauterized

have no reaction to the majority of contacts. Cauterized males occasionally responded to contact with undirected backing. This backing caused the male to lose contact with the hermaphrodite almost immediately and intermittently led to a back and forth rocking motion as the male would make contact, back and lose contact, and move forward to make contact again.

While proper contact response occurred infrequently, males with all rays cauterized were capable of occasionally producing what appeared to be posture with some backing. The duration of tail contact with the hermaphrodite was significantly reduced compared to mock ablated animals (Fig. 3.1B), most likely due to an inability to maintain the tail posture required to prolong contact with the hermaphrodite cuticle in addition to the high number of ignored tail contacts.

Residual ability to alter posture could be mediated through remaining sensory organs in the male tail, such as the HOB neuron which makes strong connections, both chemical and electrical, to the rays and many of their targets [5]. The finding that males missing the TRPP homolog PKD-2, which is expressed in B type ray neurons and HOB, have contact response defects also supports the idea that HOB might contribute to contact response [18, 19]. HOB was accordingly ablated in addition to cauterizing all rays in the *ppkd-2::ChR2-yfp* background. As in the *osm-6::gfp* background, YFP expression was absent in the cell bodies of cauterized ray neurons when examined post-assay, suggesting that entire ray neurons were killed in the cauterizing process. Males lacking all ray neurons as well as HOB responded to contact at a similar frequency as cauterized males with HOB (Fig 3.1A). Similarly, ablation of HOB did not further

reduce the amount of time that males did scan on the occasion that they responded to contact (Fig 3.1B).

Conclusions

Removal of the sensory endings of all ray neurons results in the inability to properly induce posture upon tail contact with a hermaphrodite, but leaves partially intact the ability to back. This leads to backing without direction, eventually leading to loss of tail contact with the hermaphrodite. The data suggests that though the rays appear wired to circuits hypothesized to control both posture and backing, the role of the rays is primarily to induce tail posture, while backing might additionally be mediated by other sensory organs such as the hook, PCS, or spicules. Thus contact response can be divided into two discrete components – posture change and backing. The rays are necessary and sufficient for efficient and enduring posture change and likely sufficient but not entirely necessary for backing, as backing during contact response is highly reduced in males lacking rays.

Males with all rays cauterized retain the ability to respond to contact at a low frequency. Residual posture may result from remaining sensory neurons in the tail, although elimination of the hook neuron HOB does not further reduce response. In addition, as these contacts last only a few seconds, any posture control mediated by sensory neurons other than the rays is transient and not sufficient to maintain the posture necessary for prolonged backing. This short-lived posture could also be attributed to the effect of surface tension forming the fan to the shape of the hermaphrodite cuticle,

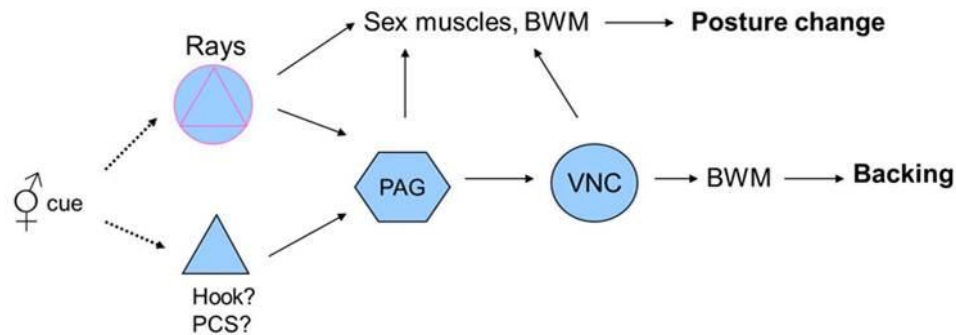


Figure 3.2. Model of Ray Mediation of Behavior

Rays, upon sensing a cue from the hermaphrodite, drive posture change and to a lesser extent backing, which together make up normal contact response. Other sensory neurons in the ventral portion of the tail such as the hook or the PCS may also participate in backing behavior. Based on wiring information (Male Wiring Project), the rays may function through interneurons in the pre-anal ganglion (PAG) and motor neurons in the ventral nerve cord (VNC) and directly to both sex-specific muscles and body wall muscles (BWM) which are common to both sexes. Triangles = sensory neurons, circles = motor neurons, hexagons = interneurons

especially as these males rarely maintain contact for more than a few seconds, so that these males might back along the cuticle by coincidence rather than with purpose.

Separation of posture and backing within contact response allows for the hypothesis of a simple model circuit (Fig. 3.2) – a hermaphrodite cue sensed upon contact by the rays leads to posture change and likely also to backing. Simultaneously, other neurons receive a cue and contribute to the propagation of backing along the hermaphrodite cuticle. These two behaviors together constitute normal contact response and allow the male to continue backing along the hermaphrodite in pursuit of copulation. The relevant interneurons, motor neurons, and muscles involved in this circuit must still be determined.

CHAPTER IV

FUNCTIONAL DISSECTION OF THE RAYS

Introduction

Our work implicates the rays collectively as the primary sensory organs mediating contact response and contributing to backwards locomotion and scanning, but allows no conclusions on the roles of subsets of rays. Rays might potentially have specialized roles such that some control posture while others mediate backwards locomotion. Alternatively, these functions could be shared amongst the rays so that any ray or pair of rays might function in these behaviors.

Previous ablation studies by Liu and Sternberg (1995) removed pairs of rays by laser ablation of progenitor cells, demonstrating that any three of the six most anterior pairs of rays could prompt contact response. This suggests that at least some of the rays share functions. However studies in other *C. elegans* behaviors reveal that when ablations are done sufficiently early in development, other cells can compensate for the physical loss of a neuron that is behaviorally significant if ablated at a later time [38]. Thus, despite the missing rays in these animals, developmental compensation by remaining rays could result in a lack of phenotype.

To confirm previous results, a portion of the ablation studies performed by Liu and Sternberg were repeated utilizing the cauterization method to eliminate rays in adult males (Table 4.1). This method has several advantages over traditional ablation of precursor cells. Elimination of mature neurons circumvents the possibility of

developmental compensation by other neurons. Additionally, destruction of the ray tips, which extend away from the main body, minimizes collateral damage to other cells making it highly probable that any phenotype observed is specifically due to loss of the ray neurons.

To expand on previous experiments, additional sets of rays were cauterized according to shared characteristics discovered since the original ablations in 1995, such as neurotransmitter subtypes and connectivity patterns (Table 4.1). The results reveal a high degree of overlap amongst the rays in that all rays appear capable of initiating contact response and instigating scanning. This redundancy is additive such that an increased number of rays prolongs the duration of scanning.

Results

To confirm the results of the experiments conducted by Liu and Sternberg, rays were cauterized in adult males according to previously described protocols. These males underwent laser surgery to destroy the sensory dendrites of the ray neurons, which ultimately led to death of the neurons themselves. Groups of three pairs were spared according to axial position along the tail – most anterior (rays 1-3), most posterior (rays 7-9), centrally located (rays 4,5,7) and distributed (rays 2,5,8). Ray 6 was eliminated in every set of cauterizations as the marker used for visualizing ray neurons, *ppkd-2::ChR2-yfp*, was not expressed in this pair.

Males possessing at least three pairs of rays, regardless of their axial position, were capable of initiating contact at a reduced but not significantly different frequency

Table 4.1. Rationale for Cauterizations

Rays remaining	Axial Position	Neurotransmitter lost	Connections lost
1-3	Anterior	DA	
3-5	Central		
4,5,7	Central	5-HT	
5,7,9		ACh	
2,5,8	Distributed		
2,8,9			Direct connections to muscle
7-9	Posterior	ACh	

The tips of the sensory rays were cauterized in adult males, sparing groups of three pairs of rays. Categories were based on stereotypical characteristics of the rays such as axial position along the tail, the neurotransmitters synthesized by the A and B neurons, and the downstream wiring targets. DA = dopamine, 5-HT = serotonin, ACh = acetylcholine

than mock males (Fig 4.1A). Amongst the subsets of cauterized rays, no one group was significantly different from another. This suggests that each spatial subset of rays is capable of triggering contact response. While frequency of contact response remained relatively intact, all groups except those possessing rays 1-3 maintained contact for much shorter periods of time than control animals (Fig 4.1B). There was no significant difference amongst the various groups of rays in their duration of scanning. While rays overlap in contact response then, they contribute additively towards scanning such that the absence of some rays does not entirely eliminate scanning but does reduce the duration of any one episode of scanning. Cauterized males also make significantly more tail contacts than uncauterized males (data not shown). This is at least in part because

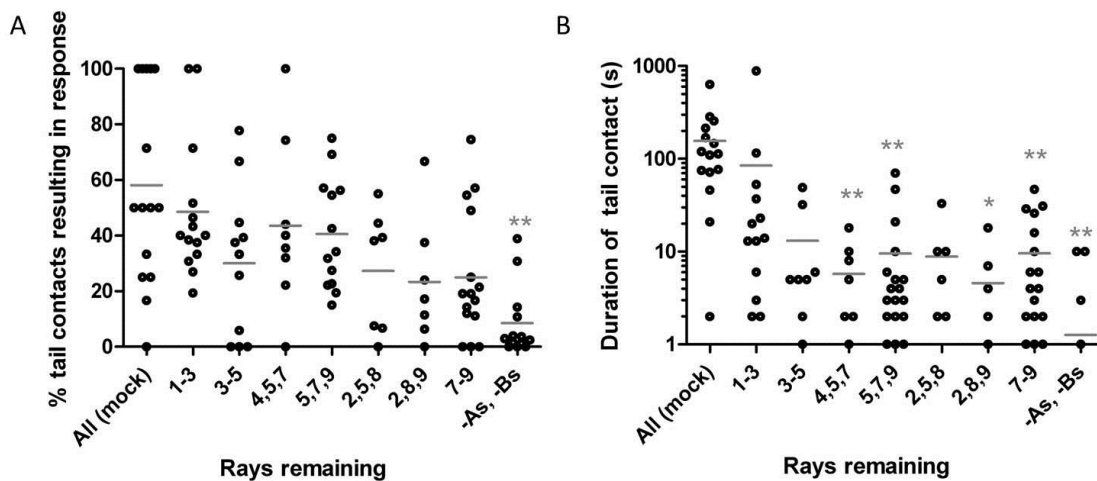


Figure 4.1. Rays Are Redundant in Contact Response but Function Additively in Scanning

A. Any combination of three ray pairs is capable of instigating contact response. Males with only rays 1, 2, and 3 lack intact dopaminergic rays. Males with rays 4,5, and 7 lack serotonergic rays. Rays 5, 7, and 9 are dopaminergic and these males lack cholinergic rays, as do males with rays 7-9. Males with rays 2, 8, and 9 lack direct connections to muscles. Each circle represents one male, gray lines indicate means. ** = p-value < 0.01, nonparametric ANOVA compared to mock animals. B. Loss of the rays significantly reduces the duration of each tail contact in cauterized animals. Each circle represents one male, gray lines indicate means. * = p-value < 0.05, ** = p-value < 0.01, nonparametric ANOVA compared to mock animals.

cauterized males have a shortened duration of scanning and so need to repeat contact response many times in a trial, while uncauterized males lose contact less often and scan for longer periods of time.

Rather than axial position, ray function might be related to the stereotyped and complex pattern of neurotransmitters synthesized in the ray neurons (Table 4.2). This includes dopamine (R5A, R7A, R9A) [2], serotonin (R1B, R3B, R9B) [16, 28], acetylcholine (R1-4A, R6A) (R. Lints, unpublished data), and a variety of neuropeptides (all RnBs, R8A) [28]. Rays can be cauterized to eliminate ray neurons known to express a particular neurotransmitter to investigate its necessity in mating behavior while

Table 4.2. Neurotransmitters in the Rays

Rays	1	2	3	4	5	6	7	8	9
A	ACh	ACh	ACh	ACh	DA	ACh	DA	NLP	DA
B	5-HT, FLPs	FLPs	5-HT, FLPs	FLPs	FLPs	FLPs	FLPs	-	5-HT

The A and B type ray neurons produce a complex, stereotypical pattern of neurotransmitters. ACh = acetylcholine, DA = dopamine, FLPs = various FMRFamide-like peptides, NLP = neuropeptide –like proteins, 5-HT = serotonin

avoiding the effects of other neurons of that neurotransmitter fate that might also be affected in mutant backgrounds.

Cauterizing all ray pairs except 1, 2, and 3 eliminates all rays known to synthesize dopamine. Eliminating all but pairs 4, 5, and 7 removes the serotonergic rays. Removing all but pairs 7, 8, and 9 or pairs 5, 7, and 9 eliminates known cholinergic rays. All combinations of ray cauterization responded to contact at a reduced but not significantly different rate compared to mock cauterized animals, and none were significantly different from each other (Fig 4.1A). Only dopamine-less animals with rays 1-3 intact scanned for a relatively normal amount of time compared to mock males, though these animals were also not significantly different from the other cauterized groups, suggesting an intermediate phenotype (Fig 4.1B). While this might imply an inhibitory role for DA in scanning, animals with only dopaminergic ray neurons – those with 5, 7, and 9 intact – display levels of scanning equivalent to all other cauterizations. Thus it seems that no one neurotransmitter plays a vital role for either

contact response or continued scanning, and that there is an overlap in the function of the various neurotransmitters in the rays.

A third potential basis of ray specialization might be through specific wiring to downstream targets. Ray neurons make many synaptic connections to each other, to interneurons, motor neurons, and directly to both sex-specific muscles and muscles common to both sexes [5]. The role of many of the male-specific interneurons is unknown. Interneurons like PVY, PVX and PVV which make synaptic connections to motor neurons (DA, DB, DD, AS, VA) or command interneurons (AVA, PVC) are likely to participate in motor behaviors such as backing [5]. Similarly, those like PDC which synapse onto muscles may control posture [5]. Close inspection of ray wiring reveals the possibility that some rays may be more suited to posture control through a large number of direct synapses to muscles, while others are potentially wired to control backing through various interneurons or motor neurons [5]. If direct muscle connections are indeed vital to posture control in contact response, then cauterizing all rays except those with no known direct connections should produce males who have difficulty producing posture upon tail contact.

To test this hypothesis, all rays except pairs 2, 8, and 9, which make no known direct muscle connections, were cauterized. These males initiate contact response at a reduced but not significantly different frequency compared to mock animals and are approximately equivalent to animals possessing only rays 7, 8, and 9 (Fig 4.1A). As both A and B type neurons in ray 7 make direct muscle connections, this suggests that these connections are not vital to posture change in contact response [5]. Similar to

other cauterizations, duration of contact response is strongly reduced compared to mock cauterized animals but is not significantly different from other combinations of cauterization (Fig 4.1B).

Conclusions

The rays exhibit a high degree of functional overlap such that no one axial position or neurotransmitter appears essential to contact response. From an evolutionary viewpoint this result might be expected. As *C. elegans* hermaphrodites self-fertilize, the only method of introducing genetic variety is through the rare males. Thus it would be highly advantageous to drive male mating behavior with an extremely robust neural network that is resistant to perturbation. Accordingly, any set of three rays appears capable of initiating contact response. This overlap might ensure that proper behavior occurs regardless of which rays are activated, another highly advantageous adaptation.

In addition to contact response, scanning appears to be induced by any set of three pairs of rays. The observation that eliminating subsets of rays reduces the duration of scanning implies that not all ray neurons are activated continuously during mating and that increasing the number of rays increases efficiency so that scanning continues no matter which rays are stimulated. Thus rays might be considered additively redundant in that all rays are capable of promoting backwards locomotion in scanning but function additively to maintain scanning behavior. This result suggests that all rays are utilized throughout scanning, which might be expected when one considers that the ray neurons

comprise almost a tenth of the male nervous system. Despite having overlapping functions then, all rays are necessary for efficient mating behavior.

Though the majority of ray neurons make synapses directly to muscles, elimination of rays that make known muscle connections does not significantly reduce posture control. This implies that ray neuron signaling through interneurons is sufficient for contact response and scanning and that direct muscle connections are not vital for this behavior. This does not eliminate the possibility that direct muscle connections are modulatory, however, and such connections might be important for fine-tuning posture during contact response and/or in the maintenance of scanning. Direct muscle synapses could also function as an alternative signaling pathway such that loss of either the interneurons or direct muscle connections would not be deleterious to behavior. The caveat should be made that the spared rays have no *known* direct connections. It is possible that connections exist but have not been identified.

Thus the ability to mediate contact response is shared amongst the rays while scanning might involve their additive action. A number of neurotransmitters can stimulate these behaviors, working through interneurons downstream of the rays. Direct muscle connections are not essential, but may contribute to fine adjustments of posture.

CHAPTER V

FUNCTIONAL DISSECTION OF RAY NEURON TYPES

Introduction

Our work points to a large overlap in ray function in contact response and scanning. Each ray, however, is composed of two ultra-structurally distinct neurons – an A type and a B type neuron, also known as RnA and RnB where n indicates the ray number. While rays as a whole appear to share roles, their ray neurons might have distinct functions in mating behavior.

Supporting this hypothesis, A and B neurons make both common and distinct downstream synaptic connections. A neurons tend to have more direct connections to muscles, while B neurons make many chemical synapses with interneurons that control backing like PVY [5, 40](A. Sherlekar and R. Lints, unpublished data). Both make numerous chemical connections to interneurons such as PVX and PVV (functions not determined), while differential connections are made to the EF interneurons, which are believed to play a role in turning – RnBs make more chemical synapses to the EFs while RnAs tend to make gap junctions [5, 40].

A and B neurons have unique molecular identities, supportive of differences in function. A neurons produce dopamine and acetylcholine, while B neurons synthesize serotonin and a variety of neuropeptides [2, 16, 28](R. Lints, unpublished data). Each neuron type possesses its own TRP channel homolog – RnAs have TRP-4, a TRPN homolog and RnBs express PKD-2 and LOV-1, both TRPP homologs [18, 20, 29, 41].

Such patterns may indicate that the ray subtypes function through different modalities. Ray neurons also express type specific α -tubulin isotypes which are required for proper neuronal function [20].

In addition, reports in the literature point to the likelihood of distinct functions for the ray neuron types. When the A neurons of rays 5, 7, and 9 were ablated, males made a wide instead of sharp ventral curl during turning. Ablation of B neurons in the same rays resulted in no behavioral phenotype, suggesting a specific role for A neurons in turning [17]. Genetic ablation of B but not A neurons significantly reduced male exploratory behavior, again suggesting a differential function for these two neuron types in a male-specific behavior[29]. Loss of specific α -tubulins in each ray neuron type leads to defects in mating behavior, as does the absence of the TRPP homologs PKD-2 and LOV-1 [18-20].

Two techniques were utilized to more thoroughly investigate the role of A and B type neurons in mating behavior. First, B neurons were specifically activated through the use of the heterologous light-inducible cation channel Channelrhodopsin-2 (ChR2)[34]. Second, neuron-specific expression of human caspases was used to genetically ablate specific ray neuron types [29, 35, 36]. The results show that both A and B type neurons are capable of inducing contact response and scanning, though with differing degrees of efficacy, but that A type neurons are specifically involved in turning.

Results

Neuron specific activation of B neurons

Driving ChR2 under the PKD-2 promoter in males raised in the presence of all-*trans* retinal (ATR), a required co-factor for ChR2, leads to depolarization of the B neurons upon blue light exposure [18, 34]. This produced a ventral tail flexure reminiscent of the tail flexure during contact response. ChR2-induced tail flexure led to a tight tail curl, as might perhaps be expected with ventral flexure in the absence of a substrate (i.e. the hermaphrodite cuticle) to press against. Tail curl was specifically the

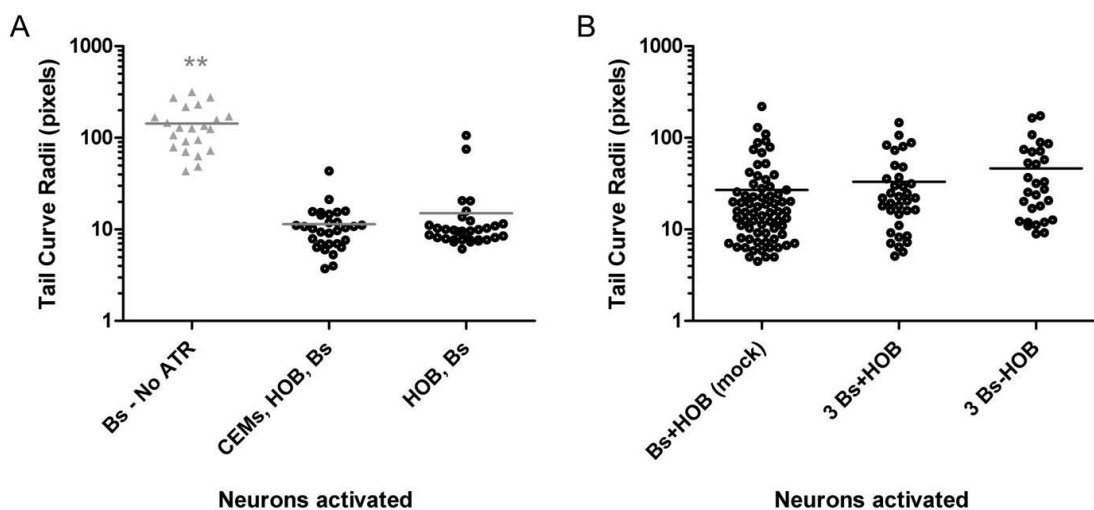


Figure 5.1. ChR2 Tail Curl Is Specific to B Neuron Activation

A. ChR2-induced tail curl requires functional ChR2 and is independent of the male-specific CEM neurons. Males grown in the absence of ATR, the required chromophore for ChR2, do not respond to blue light exposure (gray triangles). *ceh-30(n4111 n3714)* males in which the CEMs undergo improper apoptosis during development have similar tail curl to males in which the CEMs are intact. Each circle/triangle represents one male, gray lines indicate means. **= p -value <0.01 , nonparametric ANOVA, compared to males in which the CEMs, HOB, and Bs are activated. B. ChR2-induced tail curl is independent of the HOB neuron. Males in which three rays and HOB are activated are not significantly different from males in which HOB has been ablated. Neither group is significantly different from males with all B neurons and HOB intact. Each circle represents one male, gray lines indicate means. Nonparametric ANOVA.

result of ChR2 activation as transgenic males raised in the absence of ATR (and thus with non-functional ChR2) failed to tail curl in response to blue light exposure (Fig 5.1A). In addition to the RnBs, the PKD-2 promoter drives expression of ChR2 to the CEM neurons in the head and the HOB neuron of the hook [24]. To demonstrate that tail curling was independent of the CEMs, the *ppkd-2::ChR2-yfp* construct was crossed into *ceh-30 (n4111 n3714)* males in which the CEMs inappropriately undergo cell death during development [30]. No significant differences were found between tail curling in wild-type and *ceh-30* males, suggesting the CEMs do not play a major role in ChR2-induced tail curling (Fig 5.1A). Similarly, tail curl is not significantly altered when the HOB neuron is ablated, suggesting that the B neurons themselves are the driving force behind ventral tail curling in *ppkd-2::ChR2-yfp* activation (Fig 5.1B). Thus, activation of the RnBs results in a contact-response-like tail curl, implying B neuron activation is sufficient for the contact response posture change.

Our previous experiments demonstrated that multiple ray subsets are capable of prompting contact response, regardless of axial position or neurotransmitter fate and that direct muscle wiring was unnecessary. To investigate whether this overlap in function can be extended specifically to B neurons, tail curling was examined in ChR2-expressing males subjected to the previously examined cauterization categories. Both A and B type neurons in these males die in their entirety when the sensory dendrites are surgically destroyed. No combination of cauterized rays displayed a significantly weaker tail curl (Fig 5.2). This includes cauterizations by axial position (anterior, posterior, centrally-located or distributed along the tail), by neurotransmitters

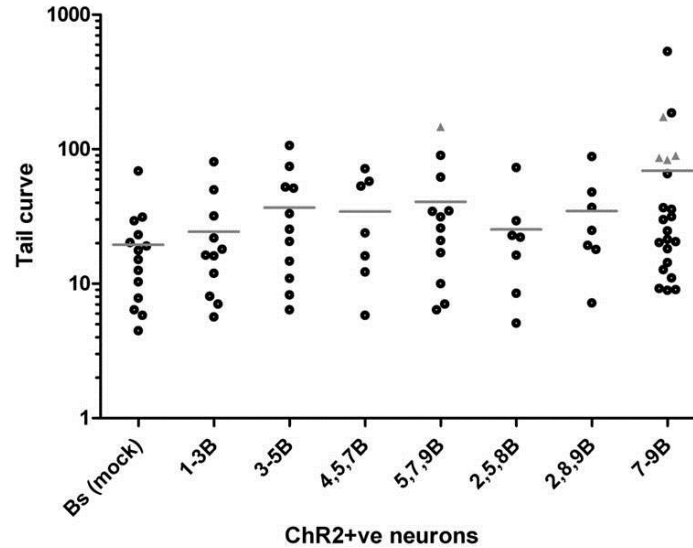


Figure 5.2. Any Three Pairs of Ray Neurons Can Induce Tail Curling

Activation of any three pairs ray neurons produces a tail curl not significantly different from activation of all rays. Rays were removed by surgically cauterizing the sensory tips with a laser. Males with rays 1, 2, and 3 intact lack dopaminergic rays. Males with rays 4,5, and 7 lack serotonergic rays. Rays 5, 7, and 9 are dopaminergic rays and lack cholinergic rays. Males with rays 7-9 also lack cholinergic rays. Males with rays 2, 8, and 9 lack direct connections to muscles. Each circle represents one male, gray lines indicate means. Gray triangles indicate males who did not respond to blue light flash. No treatment significant compared to mock animals, nonparametric ANOVA.

synthesized (removal of serotonergic rays) or cauterizations removing all known direct connections to muscles. This is in agreement with our previous findings that all combinations of cauterizations are equally capable of initiating contact response and further supports direct activation of B neurons as sufficient for contact response.

Genetically-targeted cell death of ray neurons

A second method by which to investigate the role of A and B neurons in mating is genetically-targeted cell ablation. Ray neuron types were specifically targeted to

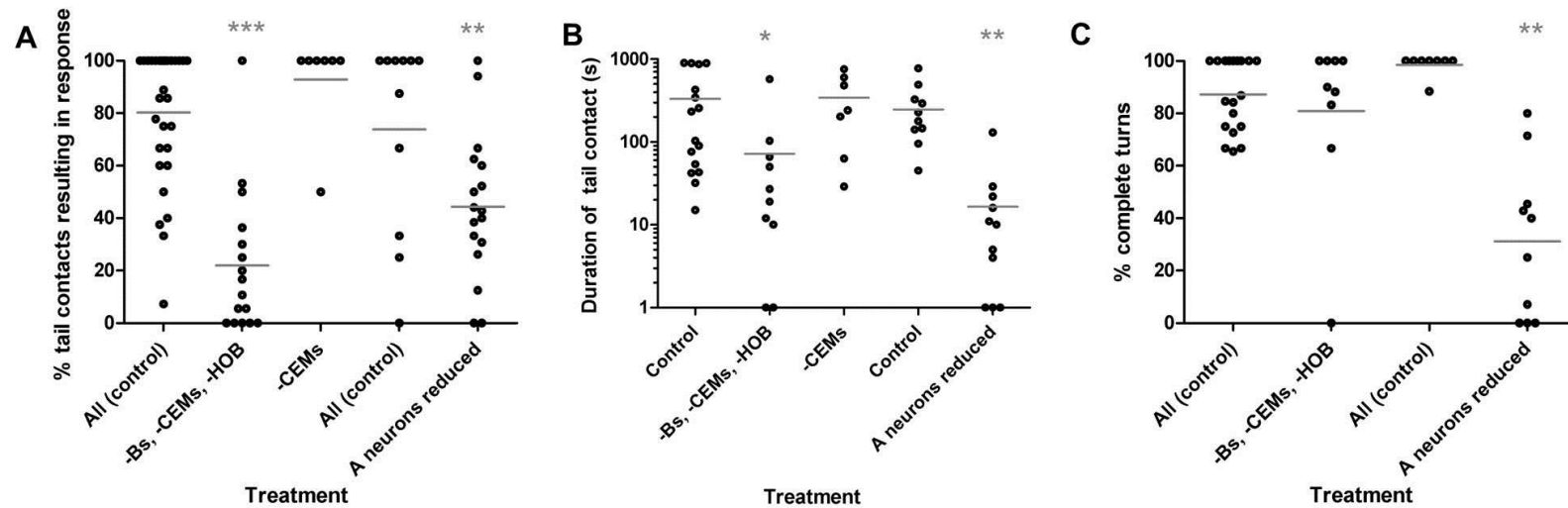


Figure 5.3. A and B Type Neurons Contribute to Common and Unique Steps of Mating Behavior

A. Both A and B type neurons contribute to contact response. Males without B neurons ignore many contacts initially but become more proficient at contact response as the mating trial progresses, eventually making normal contact response. Males lacking the CEMs alone responded to contact at wild-type levels. Males with a reduced number of A neurons stochastically make or miss contact response with no improvement during a trial. Each circle represents one male, gray lines indicate means. ** = p-value < 0.01, *** = p-value < 0.001, nonparametric ANOVA, compared to the appropriate control males. Control for B neuron ablation was *ppkd-2::gfp*. Control for A neurons reduced was *ptrp-4 + pgrd-13 + elt-2::gfp + ptrp-4::gfp*.

B. Reduction of the A neurons significantly reduces the amount of time males spend in tail contact with the hermaphrodite. Loss of the B neurons also lowers the duration of contact but to a lesser extent, while loss of the CEMs alone does not reduce duration. Each circle represents one male, gray bars indicate means. * = p-value < 0.05, ** = p-value < 0.01, nonparametric ANOVA, compared to the appropriate controls. Control for B neuron ablation was *ppkd-2::gfp*. Control for A neurons reduced was *ptrp-4 + pgrd-13 + elt-2::gfp + ptrp-4::gfp*. C. Males with reduced numbers of A neurons have difficulty completing turns. This is a major contributor to the reduced duration of tail contact in A neuron reduced males. Males lacking B neurons have no reduction in the number of complete turns once normal contact response is made. Each circle represents one male, gray lines indicate means. ** = p-value < 0.01, nonparametric ANOVA, compared to the appropriate control males. Control for B neuron ablation was *ppkd-2::gfp*. Control for A neurons reduced was *ptrp-4 + pgrd-13 + elt-2::gfp + ptrp-4::gfp*.

undergo apoptosis through the heterologous expression of caspases. Males lacking B neurons have significantly reduced rates of contact response – males ignore most contacts during a trial and several males completely failed to respond to any contact (Fig 5.3A). A number of males were able to respond to tail contact by the end of the fifteen minute trial. Though these responses often initially had weak posture or little backing, after several attempts posture and backing became wild-type in appearance, and the male appeared fully capable of scanning and turning normally afterwards, including normal contact response if he lost contact with a hermaphrodite (Fig 5.3B). Duration of contact appears reduced in large part due to initial ignored contacts and poor posture. This implies that the A neurons are capable of initiating contact response, but with reduced efficiency compared to B neurons, and that A neurons are fully capable of maintaining scanning and instigating turns. As a caveat, R6B remains in these males as the PKD-2 promoter used to drive caspase expression in the B neurons is excluded from ray 6 [18].

As the CEMs also die in males lacking B neurons, mating behavior was investigated in males in which the CEMs had been laser-ablated. These males responded to contact at a rate equivalent to mock ablated animals, implying that the CEMs do not contribute significantly to contact response (Fig 5.3A). Similarly, males lacking the CEMs scanned for a duration similar to that of mock animals (Fig 5.3B). Thus, the reduced contact response and scanning in B neuron-killed animals does not appear related to the death of the CEMs. Loss of HOB was not further explored since ablation of HOB in cauterized animals did not alter response or scanning.

Caspase expression in the A neurons led to incomplete cell death, reliably killing R3A (90% of males), R5A(90%), and R8A(85%) with more variable cell death in R2A(25%) and R6A(25%). Males with a reduced number of A neurons responded to contact less frequently compared to control animals, though this defect was not as severe as that of males missing B neurons (Fig 5.3A). A-neuron-reduced males additionally displayed a significant shortening in duration of contact, in large part due to difficulty in turning (Fig. 5.3B, Fig 5.3C). Whereas wild-type males make sharp ventral turns when they reach the end of a hermaphrodite, males lacking A neurons often backed to the end of their mate and curled their tails to the opposite side, then returned to the starting side, occasionally repeating this motion multiple times before releasing the hermaphrodite and reinitiating contact. A neuron-killed males would also occasionally swim off the hermaphrodite end without an attempt to turn, or simply let go in the middle of scanning, all behaviors rarely seen in wild-type or B neuron-ablated males.

While A neuron-reduced males were capable of locating the vulva normally, a small number displayed a slow search prodding behavior similar to that seen in males missing the HOA or HOB neuron of the hook [17]. This behavior occurred in the area of the vulva, but also on other areas of the hermaphrodite cuticle – possibly in the vicinity of the anus and excretory pore, where wild-type males will occasionally also prod as if at the vulva (R. Garcia, personal communication).

Conclusions

The data suggest that both A and B neurons are capable of instigating contact response. A neuron-contribution may be less important in contact response itself given that males lacking B neurons have a strong reduction in this behavior, but as A neuron cell death was not complete in the complementary experiment, no conclusive statement can be made. It is interesting to note, however, that loss of all but a few B neurons seems to result in little to no reduction in contact response (data not shown) while loss of only a few pairs of A neurons appears to significantly reduce this behavior.

Alternatively, B neurons might sensitize A neurons such that initial B neuron stimulation leads to A neuron-driven contact response. This fits with the observation that B neuron-ablated animals can perform all steps of mating normally once they have made normal contact response, and may also explain why males lacking only a few A neurons have noticeable mating defects. B neurons have ciliated endings that extend into the environment and so are logical candidates to encounter stimuli before the A neurons, which remain within the ray proper [4]. Further experiments with ChR2 stimulation of rays show that A neuron-activation leads to a distinct posture from B neuron-activation, and that A neuron-activation attenuates the B-neuron-induced posture (R. Lints, unpublished data). As such, activation of B neurons does not appear to act directly through the A neurons and both the A and B neurons are capable of stimulating posture.

The results also suggest a unique role for the A neurons in turning – males lacking all B neurons turn normally but males lacking even a few A neurons frequently

fail to turn. The method by which A neurons mediate turning is unknown - A neurons might function in turning through their enriched direct muscle connections, compared to B neurons [5]. However, while cauterized animals lacking all direct muscle connections rarely backed long enough to make turns, they were capable of occasionally making normal turns, suggesting direct muscle connections are not necessary for turning. Further experiments to explore the downstream targets of the rays will help illuminate the role RnAs play in this behavior. It is interesting to note that B neurons make plentiful chemical synapses onto the EF interneurons which are believed to be involved in turning, and yet males lacking B neurons can turn normally [5, 40]. A neurons (especially R2A and to a lesser extent 6A and 7A) make a number of electrical connections to the EFs, and loss of A neurons does indeed affect turning [5]. This might imply that gap junctions are more vital to ray function than chemical synapses.

Together, these results suggest that the A neurons play a vital role in male mating behavior. Loss of A neurons leads to clear deficits in contact response and turning, and milder defects in scanning and potentially vulva location. Complete elimination of the B neurons reduces only contact response, while other steps of mating behavior remain intact (except vulva location which is eliminated due to loss of the HOB neuron) [17, 19].

CHAPTER VI

CONCLUSIONS AND DISCUSSION

Successful behavior requires an organism to recognize a multitude of sensory inputs and respond with the proper motor outputs. Mating is a particularly intricate behavior that requires proper mate recognition and appropriate response to the subsequent exchange of information between individuals [42-44]. With its simple anatomy, powerful genetics, and stereotyped behavior, *C. elegans* male mating behavior is an excellent model in which to tease apart the molecular and cellular circuitry which drives a mating program.

The current studies establish the rays as the primary mediators of contact response as males lacking the sensory rays fail to posture properly upon hermaphrodite contact and only rarely back. The small amount of residual response may be attributed to remaining sensory organs on the ventral surface of the male tail such as the hook, post-cloacal sensilla, or the spicules. As the neurons of these sensory organs are highly interconnected with the ray neurons through chemical and electrical synapses, they may also share or support some of the rays' roles in mating behavior [5].

All subsets of the rays are capable of initiating contact response and promoting backwards scanning. This result is somewhat surprising as each pair of rays possesses a unique and genetically hard-wired identity which includes the pattern of neurotransmitters synthesized in the ray neurons, implying different functions for the rays [2, 16, 28] (R. Lints, unpublished data). However, as the ray neurons make synapses

to each other and to many common targets, this is certainly not improbable [5].

Signaling through common interneurons might allow for proper signaling to downstream components even in the absence of some ray inputs.

Supporting this idea, the RnBs appear to function redundantly in the promotion of contact response. Loss of all B neurons reduces contact response frequency, suggesting a role for the B neurons in this step of mating behavior. Additionally, activation of any three subsets of B neurons through ChR2 activity leads to tail curling that is similar to tail curl when all B neurons are activated. Together, the data suggest that the B neurons redundantly mediate contact response. Remaining B neurons might then function to permit initiation of contact response even in the absence of a portion of the rays.

Though elimination of B neurons reduces contact initially, males are eventually able to respond and the subsequent steps of mating are grossly normal, suggesting that A neurons are also capable of driving contact response as well as all other ray-mediated steps of mating. In support of this, even a modest reduction of the A neurons reduces the rate of contact response and leads to defects in scanning and turning. Males in which only a subset of the B neurons are killed have grossly normal mating behavior, suggesting that the A neurons function through a different mechanism than B neurons to drive contact response (R. Lints, unpublished data). Together with the deficits in scanning and turning that are not seen in males lacking B neurons, the data suggests that the RnAs might be the driving force behind ray-mediated steps of mating behavior. A role for the RnAs in mating was also observed in males lacking an A neuron-specific α -

tubulin [20]. Activation of a subset of the A neurons with ChR2 leads to a tail curl that is distinct from B neuron-induced tail posture (R. Lints, unpublished data). These data lend credence to a distinct role for A neurons in mating behavior, however incomplete death of the A neurons in the present experiments makes it difficult to untangle the role of RnAs from RnBs.

How might A and B neurons interact to drive contact response? In one scenario, B neurons might serve primarily to sensitize A neurons or even common downstream targets through the release of serotonin (5-HT) and the FLPs, both of which are known to have modulatory roles in *C. elegans* and other organisms [45-49]. In the absence of the B neurons, RnAs are capable of eventually initiating contact response but require more or prolonged stimulus to reach threshold. Once threshold is achieved, however, A neurons drive the rest of the ray-mediated steps of behavior normally.

The mechanism through which A and B neurons interact to initiate contact response is unlikely to be so simple, however. Elimination of 5-HT and the FLPs through mutation does not reduce contact response and ChR2-induced tail curl through B neuron activation in these males is similar to that of control males (P. Koo and R. Lints, unpublished data). This suggests that one or more mechanisms are functioning to permit grossly normal contact response in the absence of known RnB neurotransmitters. The possibility exists for an unidentified signaling molecule in the B neurons, especially as FLPs often function in concert with the classical neurotransmitters across phyla [46, 50-52]. In support of this idea, mutant males who genetically lack 5-HT and the FLPs have grossly normal mating behavior, compared to the reduction of response seen in B

neuron-ablated animals (P. Koo and R. Lints, unpublished data). This might imply the existence of one or more additional signaling molecules in the RnBs which are lost when the B neurons die, leading to the more severe defect. This same phenotype might be expected, however, if B neuron structure itself is important to proper signaling, regardless of neurotransmitter release. For example, B neurons might facilitate A neuron driven behavior directly through the numerous gap junctions between the two neuron types [5].

Rather than a strict role for the A neurons in mating behavior, defects in A neuron reduced animals might be due to an imbalance between A and B neuron activity. If such a balance is important, however, it appears to apply in only one direction. Males with a reduced number of B neurons have no gross mating defects, compared to males completely lacking B neurons or males with a reduced number of A neurons (R. Lints unpublished data). Further investigation into the role of RnAs and their relationship to the RnBs will likely depend on the availability of more specific promoters with which to better explore neuron ablation and activation.

Elimination of all subsets of rays which synthesize acetylcholine (ACh) or dopamine (DA) or serotonin (5-HT) did not eliminate contact response or scanning, though frequency and duration of these behaviors were reduced, respectively, for all subsets. Similarly, elimination of 5-HT and all known FMRF-amide like peptides (FLPs) in the rays through the genetic ablation of the B type neurons reduced but did not eliminate contact response initially while scanning remained grossly normal. This might suggest that no one neurotransmitter is vital for contact response and potentially that

various combinations of these neurotransmitters might be capable of generating contact response. Additional data, however, shows that the dopaminergic A neurons (R5A, R7A, R9A) specifically utilize dopamine to induce posture change in ChR2 activation (R. Lints, unpublished data). In contrast to the B neurons then at least a subset of the A neurons appear to utilize their synthesized neurotransmitter in posture control. As removal of either cholinergic or serotonergic rays did not eliminate contact response or scanning, these two neurotransmitters might both mediate A neuron function.

One mechanism by which multiple signals might be incorporated into a single response is through use of a common output signal. The ray neurons synthesize a complex pattern of neurotransmitters which includes serotonin, dopamine, acetylcholine, and a number of neuropeptides. Receptors for many of these neurotransmitters are expressed in the ray neurons themselves and/or in downstream targets of the rays such as the body wall muscles and various interneurons [23, 48, 53-55](R. Lints, unpublished data). Several of these receptors are known to be coupled to adenylate cyclase activity [53-57].

Of the four known *C. elegans* adenylate cyclases at least one, ACY-4, is expressed in a subset of the ray neurons and a number of their downstream targets (D. Greenstein, personal communication, P. Koo and R. Lints, unpublished data). Males with a loss of function mutation in ACY-4 have stochastic contact response defects as well as mild defects in turning and prodding at the vulva (P. Koo and R. Lints, unpublished data). It is not known which of the ray neuron types express ACY-4 but the stochastic contact response defect is similar to that seen in males with reduced A

neurons, though the turning deficiencies are less severe in ACY-4 mutants (P. Koo and R. Lints, unpublished data). AC5, the human homolog of ACY-4, is coupled to multiple receptors including D1 and D2 dopamine receptors and muscarinic acetylcholine receptors, family members of which are present in the *C. elegans* male mating circuit [23, 48, 53-55, 58] (R. Lints, unpublished data). Multiple signals converging on a molecule such as ACY-4 could permit ray function in the absence of individual neurotransmitters or even in the absence of entire ray neurons such as occurs during cauterization.

The two ray neuron types make many reciprocal connections with each other and often synapse to many of the same downstream targets [5]. This pattern of connectivity is significantly overrepresented in the *C. elegans* nervous system as well as the rat cortex [59, 60]. This may thus represent an important signaling motif across phyla. Though the circuitry is more compact, a similar connectivity pattern is seen in the egg-laying system of *C. elegans* hermaphrodites [61]. In this system, two pairs of neurons, the HSNs and the VCs, innervate a set of vulva muscles and make reciprocal synapses between each other [1].

In addition to a similar wiring motif, the egg-laying system also functions through a complex interplay of neurotransmitters, many of which are also synthesized in the rays. 5-HT from the HSNs primes the vulva muscles, allowing ACh from the HSNs or the VCs to stimulate egg-laying [61-63]. A similar mechanism might be envisioned in the ray circuit where the serotonergic B neurons (R1B, R3B, and R9B) promote

cholinergic A neurons (R1A-R4A, R6A) activity, either through the RnAs themselves or through common downstream targets.

Though ACh and 5-HT promote egg-laying through contraction of the vulva muscles, inhibitory functions for each have also been described within the egg-laying circuit. Both ACh and 5-HT, likely from the VCs, inhibit the HSNs [61, 64]. The muscarinic acetylcholine receptor GAR-2 mediates a portion of the inhibition of HSN and is also expressed in the cholinergic RnAs, suggesting that it may function as an autoreceptor in these neurons to modulate cholinergic signaling [65](R. Lints, unpublished data).

Application of exogenous 5-HT to different combinations of serotonin receptor mutants can either promote or inhibit egg-laying, further demonstrating that a single neurotransmitter can have both inhibitory and excitatory roles in the same behavioral circuit [66]. Similar results have been shown in males, in which exposure to exogenous serotonin leads to tail curling similar to that seen in ChR2 activation [16]. Elimination of various serotonin receptors reveals both inhibitory and excitatory roles for 5-HT in tail curling – males with mutations in the G-protein coupled 5-HT receptors SER-1 and SER-4 and the ionotropic 5-HT channel MOD-1 had reduced tail curling in exogenous 5-HT while mutations in the G-protein coupled 5-HT receptor SER-3 significantly increased tail curling [48, 53, 56, 67, 68]. SER-1 and SER-4 are likewise known to have stimulatory roles in egg-laying as well [48, 53, 69].

Dopamine has been shown to inhibit serotonergic promotion of egg-laying, potentially through a D2-type dopamine receptor and through modulation of the 5-HT

ionotropic channel MOD-1 [62, 69, 70]. At least three of the four known *C. elegans* dopamine receptors are expressed in muscles and neurons of the ray circuit (R. Lints unpublished data). While individual dopamine receptor mutant males have grossly normal mating behavior, combined defects in serotonergic and dopaminergic signaling have not been tested (P. Koo and R. Lints, unpublished data). Interestingly, B neuron-induced posture through ChR2 activity is strongly attenuated by simultaneous A neuron activation (R. Lints, unpublished data). The dopaminergic R5A, R7A, and R9A may function through mechanisms similar to those in the egg-laying system to attenuate B-neuron induced posture.

The rays and the egg-laying circuit might then utilize a complex combination of multiple neurotransmitters to drive two sex specific behaviors through similar mechanisms. These two circuits share a connectivity pattern that is over-represented in at least one instance of the mammalian brain, and thus may be an important evolutionarily conserved signaling motif [60]. As mammalian brains consist of numerous smaller circuits wired together, the ray neuron circuitry may prove to be a useful model for studying the interaction of multiple neurons driving a single output.

In total, a portion of the ray neuron circuitry in mating behavior has been diagrammed. The rays are the primary sensory organs which mediate contact response in male mating behavior, and all subsets of the rays are capable of initiating contact response. While all rays are capable of promoting backwards scanning, they function additively to prolong scanning along the hermaphrodite length. Both the A and B type neurons appear to contribute to contact response. While the exact relationship between

the two neuron types remains to be unraveled, B neurons appear to facilitate A neuron-driven behavior.

The current studies form the basis for future work on the neurons and muscles which function downstream of the ray neurons in mating behavior. In addition, knowledge of the relevant circuitry allows better identification and examination of relevant molecules such as neurotransmitters, receptors, and downstream signaling networks. Work untangling the intricate interactions between the ray neurons and the molecules they express can then inform studies in more complex models, including the human brain.

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