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Article

The Sensory Circuitry for Sexual Attraction in *C. elegans* Males

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Summary

Background: Why do males and females behave differently? Sexually dimorphic behaviors could arise from sex-specific neurons or by the modification of circuits present in both sexes. *C. elegans* males exhibit different behaviors than hermaphrodites. Although there is a single class of sex-specific sensory neurons in the head of males, most of their neurons are part of a core nervous system also present in hermaphrodites. Are the behavioral differences due to sex-specific or core neurons?
Results: We demonstrate that *C. elegans* males chemotax to a source of hermaphrodite pheromones. This sexual-attraction behavior depends on a TRPV (transient receptor potential vanilloid) channel encoded by the *osm-9*, *ocr-1*, and *ocr-2* genes. OSM-9 is required in three classes of sensory neurons: the AWA and AWC olfactory neurons and the male-specific CEM neurons. The absence of OSM-9 from any of these neurons impairs attraction, suggesting that their ensemble output elicits sexual attraction. Likewise, the ablation of any of these classes after sexual maturation impairs attraction behavior. If ablations are performed before sexual maturation, attraction is unimpaired, demonstrating that these neurons compensate for one another. Thus, males lacking sex-specific neurons are still attracted to pheromones, suggesting that core neurons are sexualized. Similarly, transgender nematodes—animals that appear morphologically to be hermaphrodites but have a masculinized core nervous system—are attracted to hermaphrodite pheromones.
Conclusions: Both sexually dimorphic and core sensory neurons are normally required in the adult for sexual attraction, but they can replace each other during sexual maturation if necessary to generate robust male-specific sexual attraction behavior.

Introduction

In the hermaphroditic nematode *C. elegans*, it is incumbent on the male to find a mate. There is considerable evidence for male-specific sexual attraction in nematodes (reviewed in [1–3]). *C. elegans* males remain on a food source if a hermaphrodite is present [4] and respond to cues released specifically by hermaphrodites [5]–clear evidence for male-specific chemosensory behavior. What are the differences between the male and hermaphrodite nervous systems that could account for sexually dimorphic behaviors? At first glance, the nervous systems of hermaphrodites and males are radically different. Out of 383 total neurons in males, 89 are sex-specific. However, most of the sex-specific neurons in the male innervate the specialized copulatory apparatus of the male, the male tail. Thus, much of the male-specific circuitry is likely to be involved in copulation rather than chemotaxis to hermaphrodites [6]. The remaining 294 neurons in the male comprise a core nervous system also found in hermaphrodites [7]. All of the sensory neurons in the head, responsible for chemotaxis of hermaphrodites to simple compounds, are also found in males. Consistent with this anatomical similarity, the chemosensory responses of the two sexes to simple compounds are to a first approximation the same [8]. In addition, males have four sex-specific chemosensory neurons in the head, the CEM neurons. These neurons undergo programmed cell death during hermaphrodite development, surviving only in males [7]. The male-specific CEMs have long been hypothesized to mediate chemosensory mate finding behavior in *C. elegans* [9]. Thus, male-specific sexual attraction could be due solely to the sex-specific CEM neurons, to subtle differences in the core circuitry, or a combination of the two.

Results

Male Sexual-Attraction Behavior

C. elegans males tax to a source of hermaphrodite pheromones and tend to remain there (Figures 1 and 2A). To characterize this response in *C. elegans*, we adapted a two-spot assay similar to those developed for studies of chemotaxis [10–12]. This assay demonstrates that *C. elegans* males tax to the peak of a pheromone gradient and remain at the source (Figures 1 and 2A). To distinguish the chemosensory behaviors elicited by pheromones from behaviors requiring tactile input, we used media conditioned by hermaphrodites (see the Supplemental Experimental Procedures and Figure S2 in the Supplemental Data available online). To generate conditioned media, we grew *C. elegans* hermaphrodites in liquid culture, removed the worms and bacteria food, and filtered the remaining liquid (Supplemental Experimental Procedures). Drops of conditioned and unconditioned media were placed on an agar test plate and allowed to soak in, and a thin coat of bacterial food was spread over the entire surface. Males on a test plate locate the source of hermaphrodite-conditioned media from a distance and tend to stay there (Figures 1, 2A, and 2B). Males respond to pheromones at a distance of up to 30 mm; analysis of single males (Figures 2C and 2D, Figure S1) shows that once an individual starts, he continuously moves toward the source until he is within 10 mm of the source center (Figure 2D, Figure S1). Attraction is specific to adult males; male L4 larvae and hermaphrodites do not

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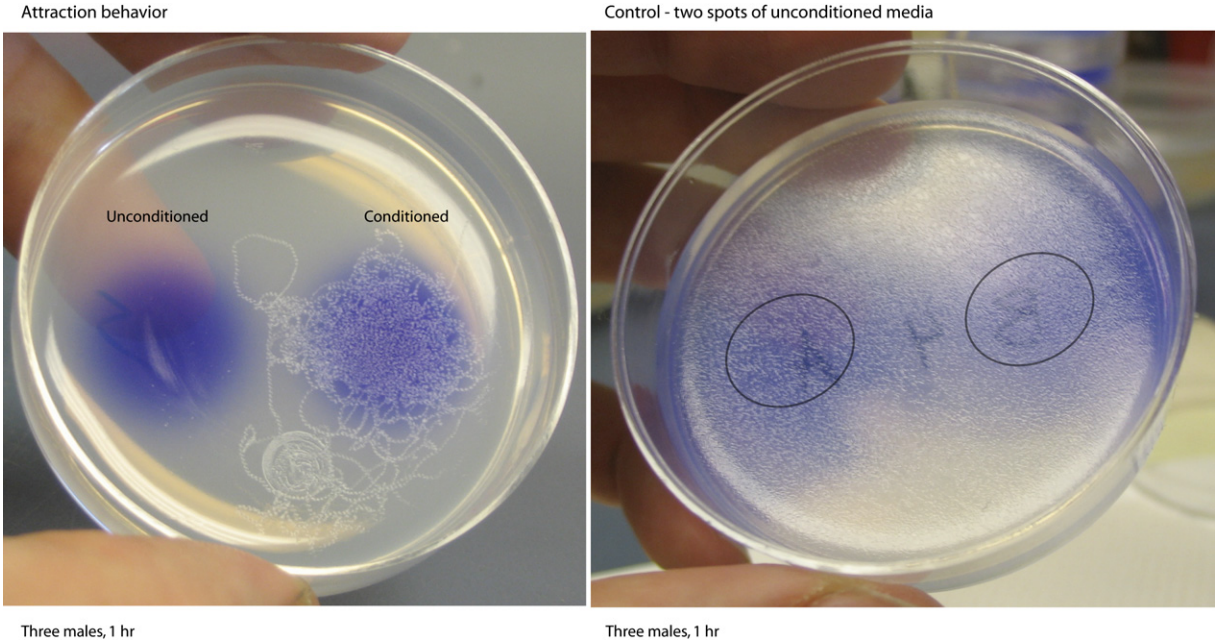

Figure 1. Male Sexual-Attraction Behavior

Males tax to a spot of hermaphrodite-conditioned media on an agar plate and tend to stay there once they have found it. Shown are photos of two-spot assays with three males each. Assays are on 50 mm (as measured) nematode growth media (NGM) agar plates spread with a thin layer of bacterial food 30 min prior to the start of the assays. Bromophenol blue to a final concentration of 0.1% (w/v) is added so that the spot on the plate could be visualized. The left panel is an assay with a control spot and a spot of hermaphrodite-conditioned media. The right panel is a control assay with two unconditioned spots. We score visual assays by the tracks males leave in the food, visible over the spot of conditioned media in the left panel. The control plate has many tracks over the entire surface of the plate. In blind assays, the plate on the left would be scored as positive for attraction, and the plate on the right would be negative.

have detectable attraction behavior (Figure 2E). Because only sexually mature males tax to hermaphrodite pheromones, and because it allows them to find their mating partners, we refer to this behavior as male sexual attraction (or simply male attraction).

Sexual Attraction Requires the TRPV Channel OSM-9

Male sexual attraction appears similar to known chemotaxis behaviors (Figure 2, Figure S1, [11, 12]). Chemosensation in *C. elegans* is mediated by ciliated neurons [13]. The proper development of ciliated sensory neurons requires the intraflagellar transport complex genes *osm-5* and *osm-6* [14, 15], and a subset—those exposed to the external environment—require a kinesin encoded by *osm-3* [14, 15]. *osm-5* and *osm-6* mutant males are partially defective for attraction (Figure 3A), so attraction is mediated by ciliated sensory neurons. The incomplete effect might be due to the potency of the attraction signal because *osm-5* and *osm-6* are only partly defective for chemotaxis behaviors to some volatile odorants [16]. *osm-3* mutants respond to volatile odorants but fail to respond to water-soluble attractants [11]. *osm-3* mutant males have no detectable defect in attraction behavior, suggesting that the attraction pheromone might be more similar to volatile odorants than to soluble signals.

In general, chemosensory behaviors in *C. elegans* require either a particular TRPV (transient receptor potential vanilloid) channel, containing OSM-9, or a particular cyclic-nucleotide-gated channel, containing TAX-2 [17]. Each channel is required for multiple, mostly nonoverlapping sets of sensory behaviors—OSM-9 for olfaction, osmosensation, and nose touch, and TAX-2 for gustation, thermosensation, and a different set of olfactory behaviors [17]. The OSM-9 TRPV channel might include additional subunits [18], and TAX-2 forms a cyclic guanosine monophosphate (cGMP)-gated channel with TAX-4 [19, 20]. To place male attraction into one of these broad sensory classes, we tested mutants in the TAX-2 and OSM-9 pathways (Figure 3B). *tax-2* and *tax-4* mutants have wild-type attraction behavior. Consistent with this, mutants in *daf-11,* which encodes a guanylate cyclase [21], also exhibit wild-type attraction. In contrast, *osm-9* mutants are defective for attraction (Figures 3B and 3C).

The defect in *osm-9* mutants is strong (Figure 3B) but often not complete (for example, *ky10* in Figure 3C), possibly indicating that OSM-9 is redundant with another TRP channel. We therefore tested mutants in the closely related channels OCR-1 and OCR-2, which are coexpressed in many of the same cells as OSM-9 [18]. The *ocr-1* and *ocr-2* single mutants have wild-type attraction behavior (Figure 3C). However, the *ocr-2*; *ocr-1* double mutant has an impaired attraction response similar to *osm-9*, but the triple *osm-9 ocr-2*; *ocr-1* mutant is no worse than *osm-9* (Figure 3C). The simplest genetic interpretation of these results is that *ocr-1* and *ocr-2* are redundant with one another and function in the same pathway as *osm-9*, consistent with the idea that OCR-1 and OCR-2 can each function as heteromer with OSM-9 [18]. Additionally, we tested mutants in the male-exclusive TRPP (transient receptor potential polycystin) channels encoded by *lov-1* and *pkd-2* (Figure 3D). *lov-1*

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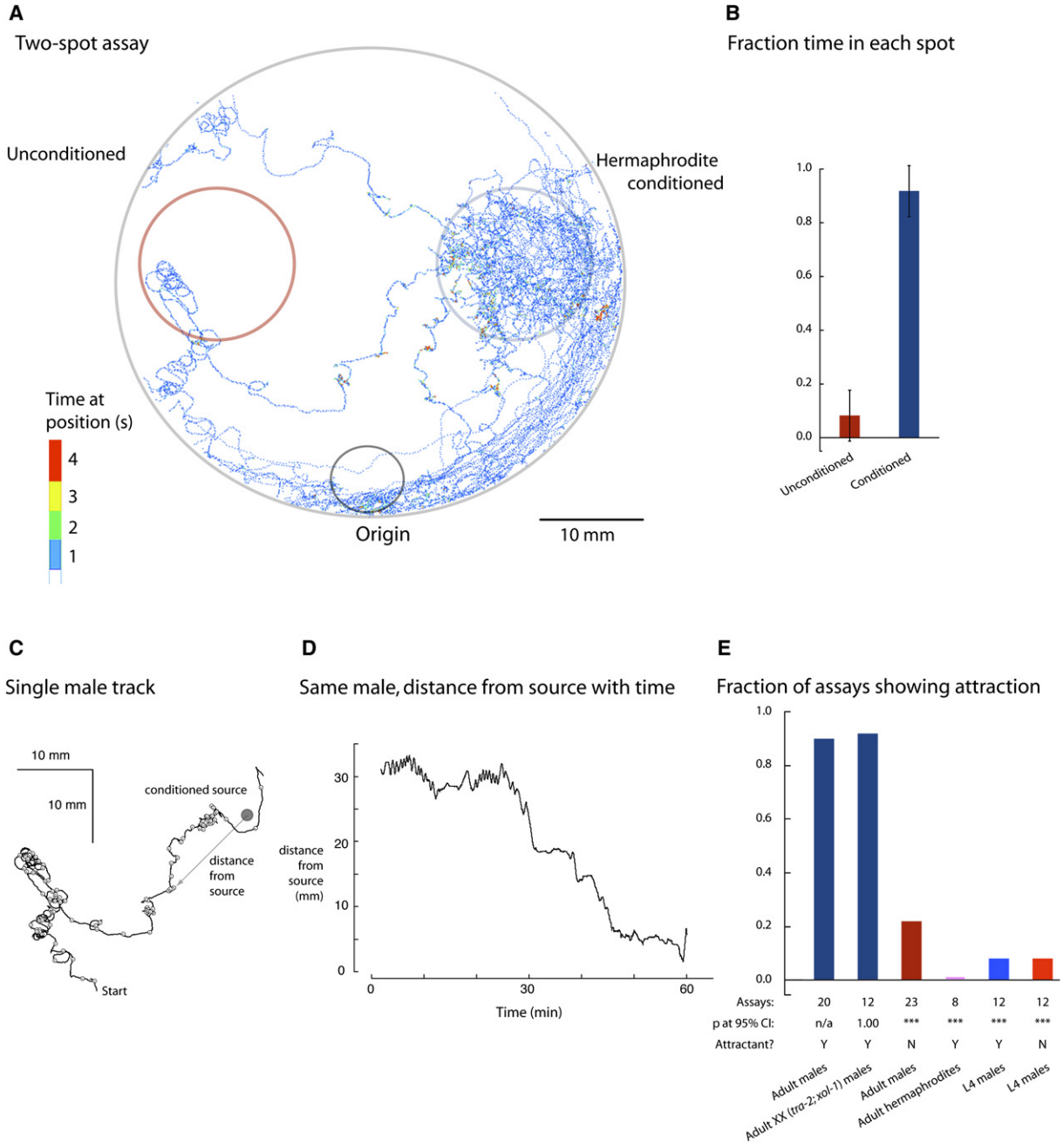

Figure 2. Sexual Attraction Is a Long-Range Chemotaxis Behavior Specific to Adult Males

(A) Video tracking of males while they are finding a spot of hermaphrodite-conditioned media. We recorded videos of ten males on a plate at 1 frame/second for 60 min and analyzed them by using computer tracking. The cumulative positions of all ten males over the entire hour are shown for one experiment, along with the boundary of the plate, the positions of the unconditioned- and conditioned-media spots, and the origin from which the males were released. A warmer pixel color indicates that worms spent a longer time at a given position.

(B) Males prefer hermaphrodite-conditioned spots. We analyzed the time males spent in either the conditioned or unconditioned spot in three independent tracking experiments. The plot shows the time the males spent in the one spot or the other normalized to the total time spent in either spot. Error bars show the 95% confidence interval of the average time for three independent tracking experiments.

(C) The track of a single male from the analysis in (A). The male suppresses looping and backing and tends to make continuous forward runs until he is within 5 mm of the center of the conditioned spot. Circles are at positions 30 s apart. Additional examples are in Figure S1.

(D) Attraction is elicited at a distance. The distance of the male in (C) from the center of the conditioned spot as a function of time is shown. The male remains at an approximately 30 mm away until 25 min into the assay, and then continuously moves toward the spot until he is within 5 mm, at about 45 min into the assay. He remains approximately 5 mm away from the center until the assay ends. Additional examples are in Figure S1.

(E) Attraction behavior is specific to adult males. The attraction responses were scored visually, blind for both the identity of animals on the plate and the identity of the spot (conditioned or unconditioned). Males tend to commit suicide by crawling off the side of the plate, so we use three males in visual assays to increase reliability. At least two assays were performed per condition on three different days, and the results combined and normalized for comparison. The total number of assays for each condition is indicated below each bar. Fisher's exact test with the Bonferroni-Holm correction for multiple comparisons was used to compare each response to wild-type ("p at 95% CI" [CI: confidence interval]); "***" indicates $p < 0.0001$.

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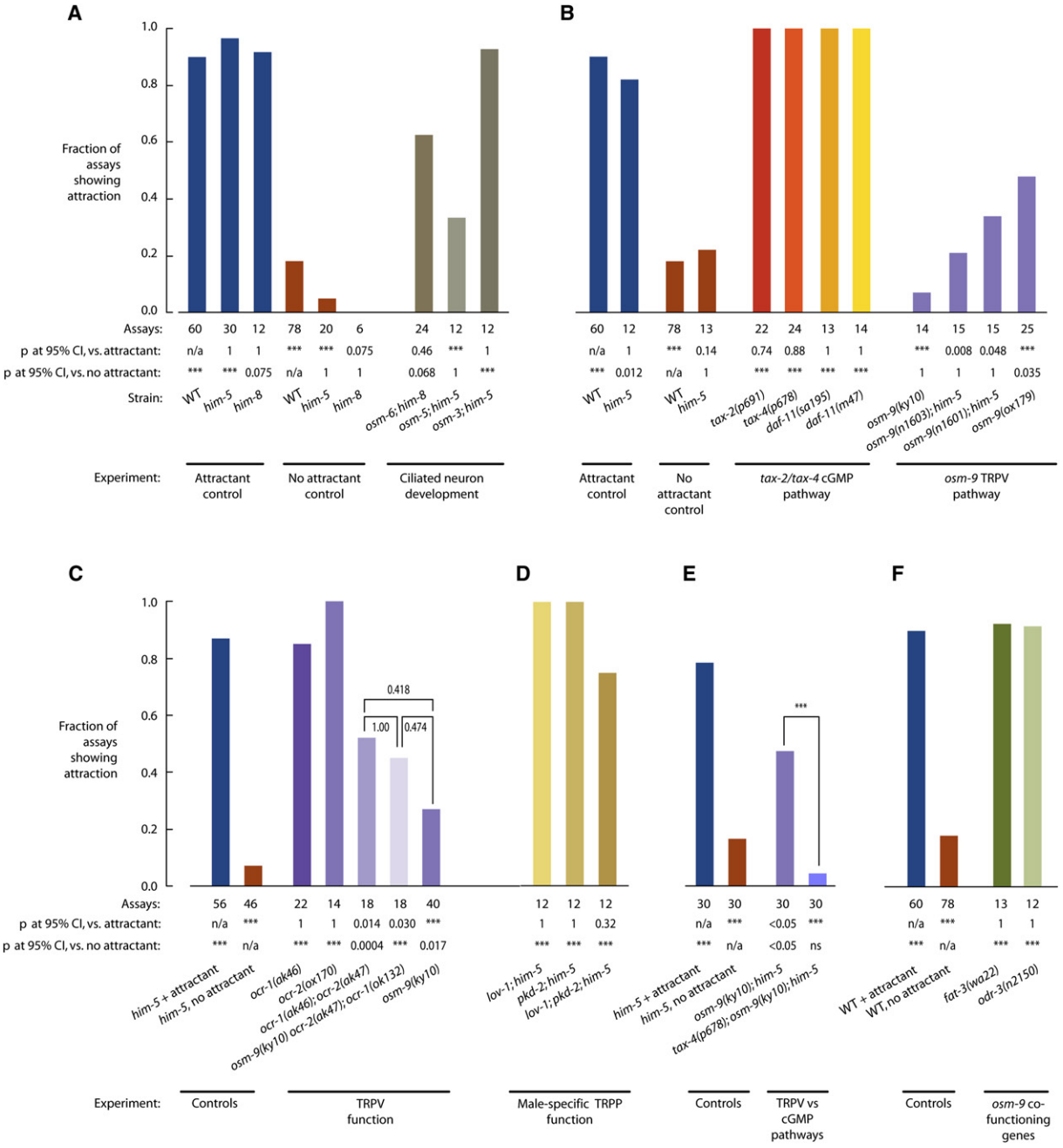

Figure 3. Male Attraction Behavior Requires a *TRPV*-Dependent Sensory Signaling Pathway, with Contributions from cGMP-Dependent Sensory Signaling

For each condition, bars represent the fraction of the indicated number of assays showing attraction behavior, scored blind, performed on at least three separate days. For each set of experiments, Fisher's exact test was used to compare the response of each strain to its corresponding *him-5*, *him-8*, or N2 Bristol conditioned media control ("p at 95% CI, attractant") and its unconditioned media control ("p at 95% CI, no attractant"). The Bonferroni-Holm correction was applied for multiple comparisons. "****" indicates $p < 0.0001$.

(A) Attraction behavior requires genes specific for ciliated sensory neuron development. The genotypes analyzed were N2 Bristol, *him-8(e1489)*, *him-5(e1490)*, *him-8(e1489) IV; osm-6(p811) V*, *him-5(e1490) V; osm-5(p813)*, and *osm-3(p802) IV*.

(B) Attraction behavior is impaired in *osm-9* mutant males, but not in single mutant males defective for cGMP signaling mediated by *tax-2* and *tax-4*.

(C) Attraction requires *ocr-1* and *ocr-2*, which are redundant with one another and act in the same pathway as *osm-9*. Fisher's exact test with Bonferroni-Holm correction for multiple comparisons was used for pairwise comparisons between the *ocr-2 osm-9*; *ocr-1* triple mutant, the *ocr-2*; *ocr-1* double mutant, and the *osm-9* single mutant.

(D) Attraction behavior does not require the male-specific TRPP channel subunits encoded by *lov-1* or *pkd-2*. Genotypes analyzed were *him-5(e1490)*, *lov-1(sy582); him-5(e1490)*, *pkd-2(sy606); him-5(e1490)*, and *lov-1(sy582); pkd-2(sy606); him-5(e1490)*.

(E) TAX-4-dependent cGMP signaling contributes to attraction behavior in the absence of OSM-9. Fisher's exact test with the Bonferroni-Holm correction was used to compare the *osm-9(ky10)* single mutant and the *tax-4(p674); osm-9(ky10)* double mutant.

(F) Genes that often function in *osm-9* pathways are not required for attraction. *fat-3* encodes an omega-3 lipid desaturase required for the synthesis of polyunsaturated fatty acids, which in some cases are required for function of OSM-9. *odr-3* encodes a G_{α} subunit that in some cases is thought to activate OSM-9.

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and *pkd-2* are expressed in specialized sensory neurons not present in hermaphrodites, including the CEM neurons, the hook neuron HOB, and the sensory ray neurons, and are required for mechanosensory steps of male mating [22, 23]. It might be expected that these genes are required for other male-specific sensory behaviors, such as attraction. However, *lov-1* single-, *pkd-2* single-, and *lov-1; pkd-2* double-mutant males have unimpaired attraction behavior (Figure 3D). This is perhaps not surprising, given evidence leading to the hypothesis that TRPP channels directly sense movement or mechanical stimuli [24] rather than function in chemosensation. Because OSM-9- and TAX-4-containing channels are in some cases required for different functions in the same neuron (AWC, for example [17]), we tested the behavior of *osm-9; tax-4* double-mutant males (Figure 3E). Although a *tax-4* mutation has no effect on its own, it eliminates the remaining attraction behavior of an *osm-9* mutant ($p < 0.001$, Figure 3E). Thus, TAX-4 cGMP signaling is not absolutely required for male sexual attraction, but it might act in some neurons to assist OSM-9 signaling, and it accounts for the remaining behavior in an *osm-9* mutant.

Mutants with defects in genes that typically function with the OSM-9 TRPV channel have normal attraction behavior (Figure 3F). In nematodes, OSM-9 TRPV channels are thought in some circumstances to be activated by G protein signaling [24]. The *C. elegans* G_{α} subunit encoded by *odr-3* is expressed in the *osm-9* olfactory neurons AWA and AWC and has phenotypes similar to *osm-9* [25]. However, *odr-3* mutant males have normal attraction behavior. Sensory G proteins might activate OSM-9 by mobilizing specific polyunsaturated fatty acids (PUFAs), the synthesis of which depend on an omega-3 lipid desaturase encoded by the *fat-3* gene [26]. *fat-3* mutants have sensory phenotypes similar to *osm-9* and can be rescued by the exogenous application of PUFAs in an *osm-9*-dependent manner [26]. However, *fat-3* mutant males have normal attraction behavior (Figure 3F). We speculate that there might be additional cofunctioning genes that we have not identified, or, more intriguingly, that OSM-9 might have a unique function in male attraction.

Sexual Attraction Requires Both Male-Specific and Core Sensory Neurons

To determine the sensory neurons required for male attraction, we performed laser-ablation experiments. Likely candidates to test by laser-ablation were neurons that express *osm-9*. In hermaphrodites, *osm-9* is expressed in the neuron pairs AWA, AWC, ASE, ADF, ADL, ASG, ASH, ASI, ASJ, ASK, IL2, OLQ, PVD, PHA, and PHB [17]. In males, by using the canonical *osm-9* transcriptional fusion (*osm-9::gfp5*, [17]), we see additional *osm-9* expression in male-specific neurons in the tail, possibly the RnB and HoB neurons, and in the male-specific CEM neurons in the head (data not shown, schematic in Figure 4A). It has long been hypothesized that the CEMs are sensory neurons that mediate male attraction to hermaphrodites [9]. Accordingly, we ablated the two pairs of CEM neurons in L4 males (Figure 4B) and assayed the operated animals as adults in single-animal, numerically scored attraction assays in parallel with mock-ablated controls. Attraction is greatly impaired in males with all four CEM neurons ablated. Males with one or more CEM neurons exhibit attraction behavior, although the response might be reduced (not shown). This indicates that the male-specific CEM neurons are required for sexual-attraction behavior.

Attraction has characteristics of a long-range chemosensory behavior (Figures 2C and 2D, [10–12]), implicating olfactory or gustatory neurons in the response. Of the *osm-9*-expressing neurons, AWA and AWC are major olfactory neurons, and ASE is a major gustatory neuron [10–12]. Notably, *osm-9* is required for all known sensory functions of AWA [24]. Accordingly, we ablated either the AWA or AWC pair in L4 males (Figure 4B). Attraction is greatly impaired in adult males lacking either AWA or AWC. In contrast, males in which the ASE pair is ablated have an attraction response that is not significantly different from mock controls (Figure 4B). Thus, in addition to the CEM neurons, both the AWA and AWC neurons, but not the ASE neurons, are required for male attraction behavior.

Male Sensory Neurons Can Compensate for One Another during Development

The ablation of the CEM, AWA, or AWC neurons impairs attraction behavior only when we perform the ablations on L4 larvae. The ablation of these neurons one set at a time in L3 larvae, earlier in development, did not detectably impair male attraction (Figure 4C). This is not simply due to a longer recovery period, because L4-ablated animals given the same amount of time to recover as L3-ablated animals (2 days) remain defective for attraction (data not shown). On this basis, we hypothesized that the male nervous system can compensate for the loss of the CEM, AWA, or AWC neurons if they are absent in the L3 stage or earlier. To test this hypothesis, we simultaneously ablated the CEM, AWA, and AWC neurons in L3 males. Adult animals with all three sets of neurons ablated at the L3 stage have impaired attraction behavior (Figure 4C). The attraction response of these animals is not significantly different than the single-set L4-ablated animals ($p > 0.05$, one-way analysis of variance [ANOVA] with Dunnett's multiple comparison test). Thus, in L3-ablated males, the CEM, AWA, and AWC neurons compensate for one another; the remaining sets of neurons adjust for the ablated set. In L4-ablated males, the nervous system no longer has this capacity.

Genetic mutants in which the CEM, AWA, or AWC neurons have lost their identity or are lost altogether should be equivalent to an ablation very early in development, and so should mimic the L3 ablation of these neurons. Accordingly, we tested strains with mutations that specifically eliminate the CEM neurons (*ceh-30,* Barbara Conradt and Phillip Grote, personal communication), the AWA neurons (*odr-7* [27]), or the AWC neurons (*ceh-36* [28, 29]). Consistent with our L3 ablation results, single and double mutants had no significant effect on male sexual-attraction performance (Figures 4D and 4E), but the *ceh-30 odr-7 ceh-36* triple mutant has strongly impaired male attraction behavior (Figure 4F). Notably, the ASE neurons are always present in the triple mutant, indicating that they are not required for attraction, and so contribute very weakly, if at all. Thus, the genetic removal of the CEM, AWA, and AWC neurons

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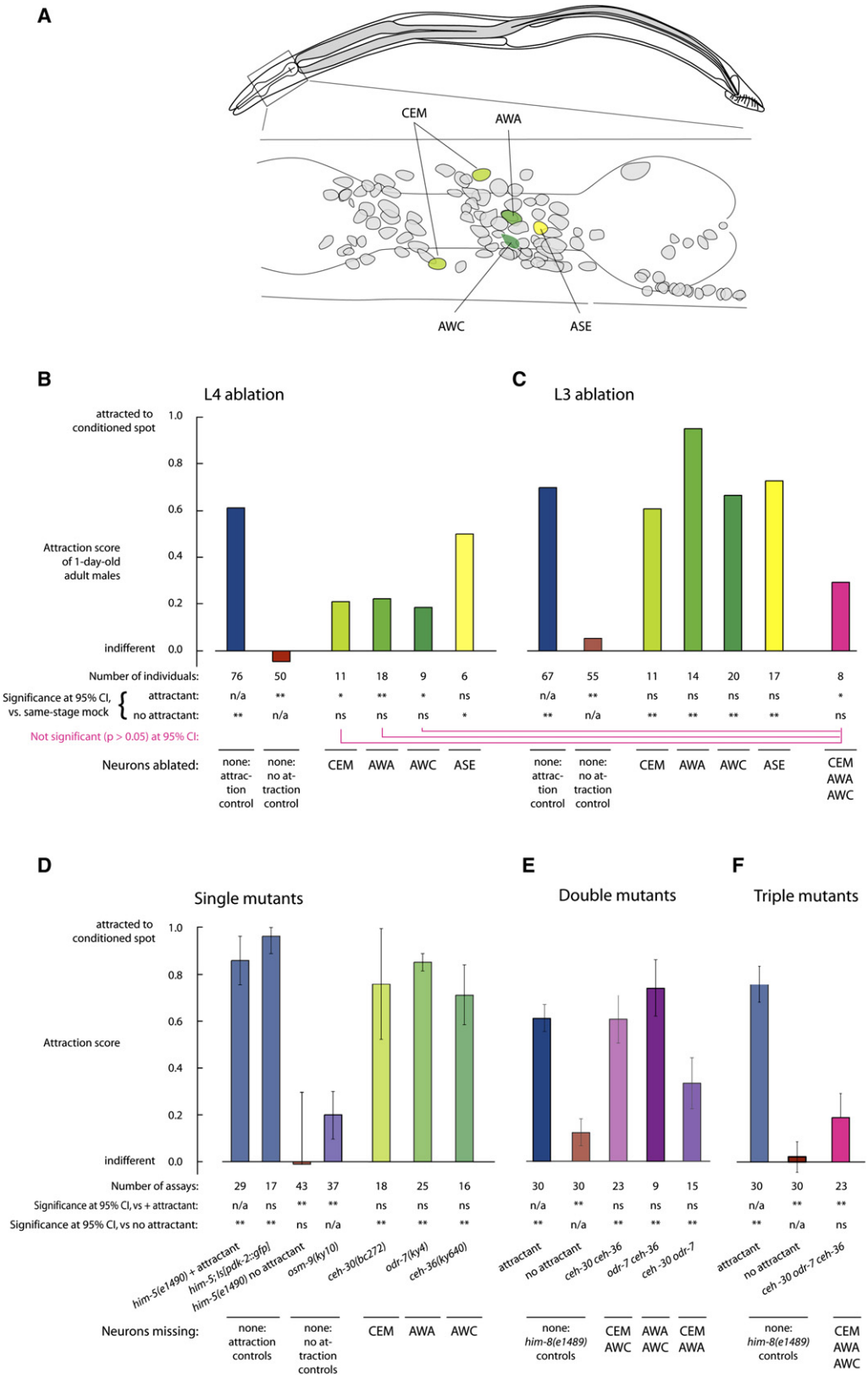

Figure 4. Male Sexual Attraction Requires the AWA and AWC Olfactory Neurons and the Male-Specific CEM Neurons, All of which Can Compensate for One Another

(A) Schematic of male neuroanatomy. A diagram of a *C. elegans* male with the head ganglia shown enlarged, indicating the anatomical position of the CEM, AWA, AWC, and ASE neuron nuclei. Only the left side is shown; bilaterally symmetric neurons are found on the right side. Diagrams are adapted from [33, 36].

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recapitulates the removal of these neurons by laser ablation, demonstrating that they are the main sensory neurons required for male attraction behavior and supporting the conclusion that they compensate for one another if removed early in development.

Sexual-Attraction Behavior Depends on the Ensemble Output of the AWA, AWC, and CEM Neurons

To determine whether additional *osm-9*-expressing neurons might contribute to male sexual attraction, we rescued *osm-9* in combinations of the AWA, AWC, and CEM neurons in an *osm-9(ky10)* mutant background by using cell-specific promoters (Figure 5), and we assayed the attraction behavior of the rescued strains. The expression of *osm-9* in each single neuron class and in the CEM + AWC combination did not restore attraction behavior; these males were not significantly different from the *osm-9(ky10)* control. Expression in the CEM + AWA and AWA + AWC combinations resulted in attraction behavior that was not significantly different either from the *osm-9(ky10)* or from *him-5* controls, suggesting intermediate behavioral performance. Concurrent expression in the CEM, AWA and AWC neurons completely restored behavioral performance, and was comparable to a strain in which *osm-9(+)* is expressed in the entire nervous system. The fully rescued males are not significantly different from the *him-5* control and are significantly different from the *osm-9(ky10)* control. These results indicate that fully functioning AWA, AWC, and CEM neurons are sufficient for male attraction behavior; if other *osm-9*-expressing neurons contribute, their contribution must be minor. Also, because the *pkd-2* promoter used to drive CEM expression in these experiments only comes on strongly in the adult [22, 23], OSM-9 must function in—or maintain the function of—the adult circuit, and so is most likely not required for the developmental wiring of the male sexual-attraction sensory circuit. Finally, these results indicate that AWA, AWC, and CEM function is nonredundant—it is necessary for all three classes to be active to achieve full behavioral rescue, consistent with the L4 ablation results.

Reversing Sexual Preference

Only males are attracted to hermaphrodite pheromones. Hermaphrodites could in principle be attracted to pheromones, but they are not. What is the site of the sex specificity of male attraction behavior? We considered two possibilities: One is that sex-specific hormonal signals from the gonad or other nonneuronal tissues influence the function of the core nervous system, and the other is that sexual attraction is intrinsic to the male circuitry and so depends only on the sex of the nervous system. To distinguish these possibilities, we masculinized the nervous system of hermaphrodites. The overexpression of *fem-3* throughout an entire XX animal during development is sufficient to masculinize the animal and turn off hermaphrodite-specific genes [30]. We therefore expressed the *fem-3* complementary DNA (cDNA) from the nervous-system-specific *rab-3* promoter to generate animals that were hermaphrodites with respect to karyotype and overt morphology but expressed *fem-3* at high levels in the nervous system. The overexpression of *fem-3* in the nervous system was sufficient to induce survival of the male-specific CEM neurons in ~1% of animals, revealed by a P(*pkd-2)::gfp* reporter (data not shown). Male-specific neurons in the tail were never observed because the progenitors of these cells differentiate as skin cells in hermaphrodites rather than neurons as in males [31–33] and never express neuronal genes such as *rab-3*. Hermaphrodites with masculinized nervous systems have robust attraction behavior that is indistinguishable from control males (Figure 6) and significantly different from nonmasculinized hermaphrodites. This result demonstrates that the sex-specificity of male attraction behavior is determined solely by the sexual identity of the nervous system. Masculinization of the core neurons is sufficient for attraction to hermaphrodites.

Discussion

Why are males attracted to hermaphrodite pheromones, but hermaphrodites not? Our results demonstrate that the sex specificity of male attraction depends only on

(B) L4 ablation of either the AWA, AWC, or CEM neurons severely impairs attraction behavior in adult males. Bars represent the average score of all ablations at each condition. One-way ANOVA with Dunnett's multiple comparison test was used to compare ablated males to control animals tested with attractant or no attractant ("Significance at 95% CI, vs. mock"); "**" indicates $p < 0.01$, "*" indicates $p < 0.05$, and "ns" indicates $p > 0.05$ (not significant).

(C) L3 ablation of any single class of the AWA, AWC, or CEM neurons has no affect, but the simultaneous ablation of all three classes severely impairs attraction in adult males. Data representation and statistics are as in (B). Additionally, Dunnett's multiple comparison test was used to compare the CEM, AWA, AWC multiple L3 ablation to the L4 single ablations in (B) ($p > 0.05$).

(D) Attraction behavior of single mutant males deficient in the AWA, AWC, or CEM neurons is unimpaired. The following strains were tested: *him-5(e1490)*, MD549 *him-5(e1490) V; bcIs9[pkd-2::gfp] lin-15(n765ts) X*, MD1400 *him-5(e1490) V; bcIs9 ceh-30(bc272) X,* CX5922 *ceh-36(ky640) X ; kyIs140[str-2::gfp] I*, and CX4 *odr-7(ky4)*. Bars represent the average of three experiments on different days, and error bars indicate the standard error of the mean (SEM). One-way ANOVA with Dunnett's multiple comparison test was used to compare each strain to *him-5(e1490)* tested with attractant ("significance at 95% CI, vs. *him-5* + attractant"), and to *osm-9(ky10)* ("significance at 95% CI, vs. *osm-9(ky10)*"). "**" indicates $p < 0.01$, "*" indicates $p < 0.05$, and "ns" indicates $p > 0.05$ (not significant).

(E) Attraction behavior of double-mutant males in which pairs of the AWA, AWC, and CEM neuron class are affected is unimpaired. The following strains were tested: *him-8(e1489)*, EG4703 *him-8(e1489) IV ; bcIs9[P(pkd-2)::gfp] X ceh-30(bc272) X ceh-36(ky640) X ; oxEx1025[P(pkd-2)::gfp, P(ceh-36)::gfp, P(unc-17)::mCherry]*, EG4704 *him-8(e1489) IV ; odr-7(ky4) X ceh-36(ky640) X ; oxEx1028[P(odr-10)::gfp, P(ceh-36)::gfp, P(unc-17)::mCherry],* and EG4709 *him-8(e1489) IV ; bcIs9[P(pkd-2)::gfp] X ceh-30(bc272) X odr-7(ky4) X; oxEx1037[P(pkd-2)::gfp, P(odr-10)::gfp, P(unc-17)::mCherry].* Data representation and statistics are as in (D).

(F) Attraction behavior of triple-mutant males in which the AWA, AWC, and CEM neurons are simultaneously affected is severely impaired. We tested the sexual attraction behavior of *ceh-30(bc272) odr-7(ky4) ceh-36(ky640)* triple-mutant males in three sets of experiments on different days in three independently isolated mutant lines. We verified that the neurons predicted to be affected were indeed absent by checking for the absence of green fluorescent protein (GFP) expression from reporters specific to each cell. The genotype tested was *bcIs9[P(pkd-2)::gfp] X ceh-30(bc272) X odr-7(ky4) X ceh-36(ky640) X ; oxEx1023[P(pkd-2)::gfp, P(odr-10)::gfp, P(ceh-36)::gfp, P(unc-17)::mCherry]*. Data representation and statistics are as in (D).

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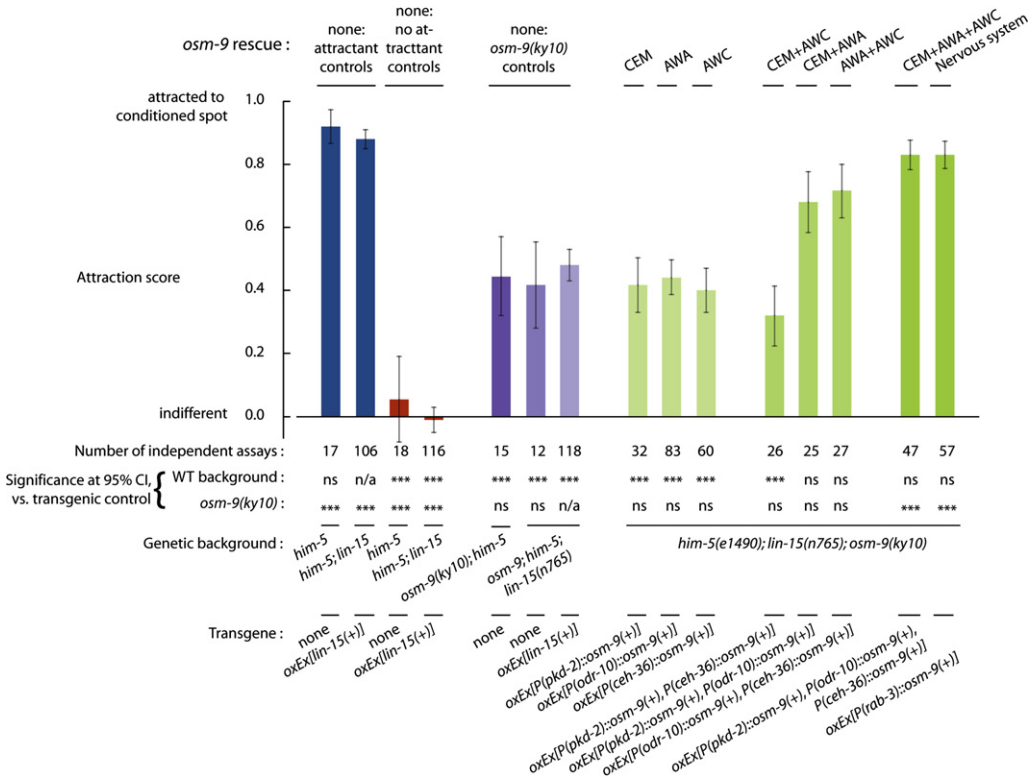

Figure 5. *osm-9* Rescue in the CEM, AWA, and AWC Neurons Completely Restores Attraction Behavior

We expressed a cDNA encoding the wild-type *osm-9* gene in the indicated combinations of the CEM, AWA, and AWC neurons in an *osm-9(ky10)* genetic background. Violet bars indicate *osm-9(ky10)* controls with no rescue. Light green bars show the behavior for expression in each single class, medium for pairs of classes, and dark green for all three classes or the entire nervous system. Bars represent the average of the total number of assays indicated; error bars indicate the SEM. One-way ANOVA with Dunnett's multiple comparison test was used to compare each strain to a *him-5(e1490)* transgenic control strain (EG4222 or EG4223, Table S1) tested with attractant ("significance at 95% CI, vs. transgenic control") and to *osm-9(ky10)* transgenic control strain (EG4244 or EG4255) tested with attractant ("significance at 95% CI, vs. *osm-9(ky10)*"). "***" indicates $p < 0.001$, and "ns" indicates $p > 0.05$ (not significant).

the sex of the nervous system, so the question further simplifies to the following: What are the differences between the male and hermaphrodite nervous systems that give rise to attraction behavior? Our results identify differences at both the molecular and neural levels.

At the molecular level, it is likely that there are sex-specific inputs into general OSM-9-dependent sensory signaling. Because OSM-9 is required for a number of sensory behaviors [17], the OSM-9/OCR-1/OCR-2 channel most likely mediates general sensory signal transduction downstream of a male-specific pheromone receptor or receptors in the adult sexual-attraction circuit. Our results indicate that the attraction defect in *osm-9* mutant males is due to a partially functioning sensory system rather than developmental differences within the population. The strong effect of *osm-9* mutations shows that OSM-9-dependent signaling is the major pathway, but because *osm-9* mutations do not completely abolish sexual attraction, there must be additional sensory signal-transduction pathways that contribute. One pathway appears to be a TAX-4-dependent cGMP signaling system because attraction behavior is completely abolished in a *tax-4*; *osm-9* double mutant. However, *tax-4* or *tax-2* mutants alone do not impair male attraction behavior, even at very dilute pheromone concentrations (J.Q.W. and T.J.N., unpublished data), so the relative contribution of a TAX-4 cGMP pathway in the presence of OSM-9 is unclear. It could perhaps have a sex-specific modulatory function on OSM-9-dependent signaling, or the converse. Regardless, it is likely that *osm-9* mutations affect male sexual attraction because they impair but do not abolish function of all of the sensory neurons required for attraction—AWA, AWC, and the CEMs.

Normally, the AWA, AWC, and CEM neurons are all required for male-specific sexual attraction, as shown by the L4 ablations. It appears as if attraction is not completely abolished in the ablated animals; however, their response is not significantly different from mock controls tested in assays without conditioned media, so the effect is strong, if not complete. This demonstrates that the AWA, AWC, and CEM classes of neurons are the major sensory neurons mediating male sexual attraction. In contrast to L4 ablation, the elimination of any single set of the AWA, AWC, or CEM neurons in L3 larvae or earlier, by either ablation or by mutation, does not impair sexual attraction. However, the concurrent removal of all three classes severely impairs attraction behavior, demonstrating that these are indeed the relevant sensory neurons. The male nervous system compensates for the early loss of neurons that are normally required in the adult.

Why does compensation not occur for the L4 ablations? The answer might have to do with the time during development when neurons are wired into a functional

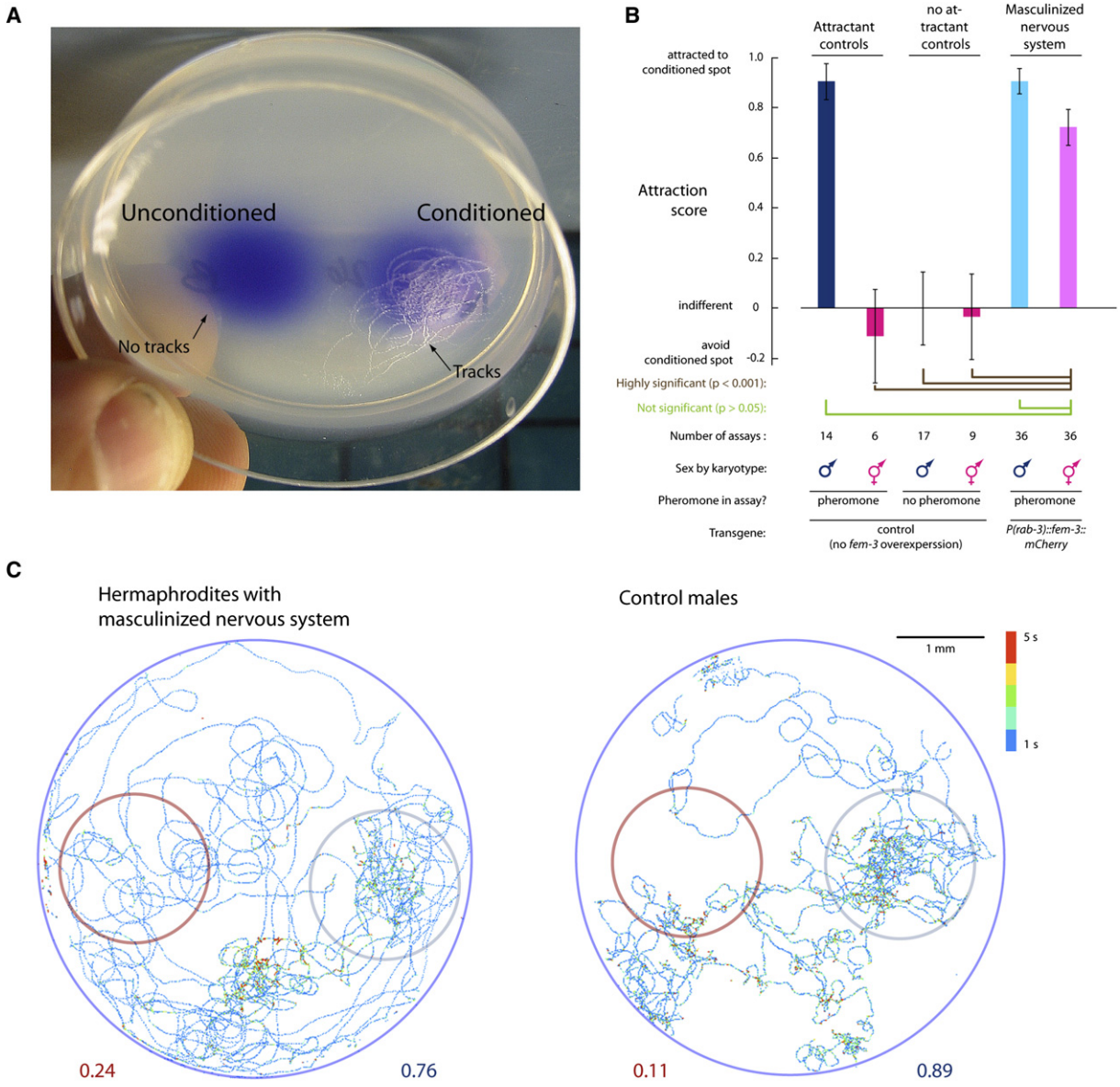

Figure 6. The Sex Specificity of Attraction Behavior Depends Solely on the Sexual Identity of the Nervous System

We masculinized the hermaphrodite nervous system by using the *rab-3* promoter to express a cDNA encoding the wild-type *fem-3* gene specifically in the nervous system. We assayed the attraction behavior of the transgenic XX hermaphrodites and, as a control, their XO male siblings. (A) Attraction assay of three hermaphrodites with a masculinized nervous system. Reprogrammed hermaphrodites tax to the conditioned media spot and remain there, as shown by the tracks they leave on the plate. The elapsed assay time is 1 hr. The plate diameter is 50 mm.

(B) Attraction behavior in hermaphrodites with a masculinized nervous system is comparable to males. Attraction behavior was assessed in numerically scored, blind attraction assays. We assayed both XX and XO karyotypes in transgenic control strains (no *fem-3* expression; EG4222 or 4223) in the presence and absence of pheromone attractant. We compared these controls to both XO and XX individuals in strains expressing *fem-3* in the nervous system using the *rab-3* promoter. Bars represent the average of the total number of assays indicated; error bars indicate the SEM. One-way ANOVA with Bonferroni's multiple comparison correction was used to compare the response of each strain with all others.

(C) Video tracking of control males and reprogrammed hermaphrodites while they are finding a spot of hermaphrodite-conditioned media. We recorded videos of eight animals on a plate at 1 frame/second for 60 min and analyzed them by using computer tracking. The cumulative positions of eight hermaphrodites with a masculinized nervous system are shown on one plate and compared to the positions of eight sibling males on another. The boundary of the plates and the positions of the unconditioned- (red circle) and conditioned-media (blue circle) spots are indicated. A warmer pixel color indicates that animals spent a longer time at a given position. Numbers indicate the relative amount of time animals spent in the unconditioned (red) spot compared to the conditioned (blue) spot for each tracking experiment.

sensory circuit. During the L4 stage, the male nervous system undergoes dramatic remodeling to form the adult connections [31, 32]. It is likely that the attraction sensory circuit is wired at this time. L4 ablations could remove neurons from the circuit after the adult connections have been formed, or as they are forming. The male nervous system might not be able to compensate for the loss of a neuron after the final adult connections are formed. L3 ablations (and developmental mutations) could remove neurons before the adult connections have been formed. The remaining neurons somehow detect the absence and compensate for the missing

neurons during final wiring, perhaps by strengthening their connections to a common interneuron or by invading postsynaptic fields normally occupied by the missing neurons. Double mutants with only a single class of sensory neurons remaining still have robust attraction behavior, indicating that one set is sufficient. Similar strong compensatory effects allow the hypothalamic circuitry that normally promotes feeding in mice to be ablated in neonates with no affect on food intake [34]. Acute ablation in adults, however, causes mice to starve themselves [34, 35]. The neural circuitry that controls such fundamental behaviors such as sex and feeding seems to be particularly robust.

Losing a class of neurons is generally worse than losing their *osm-9*-dependent function. For example, males in which the CEM neurons have been ablated in L4 larvae have severely impaired attraction behavior, but *osm-9* mutant males in which *osm-9* is rescued in the AWA + AWC neurons have intermediate attraction behavior. One explanation is that an additional sensory signaling pathway in the CEM and AWC neurons—most likely a TAX-4-dependent pathway—contributes to the male attraction response. This is consistent with published results showing that TAX-4 is required for the primary olfactory functions of AWC [19, 20]. In one case, L4 ablation and rescue are equivalent: Males missing AWA by ablation and males missing *osm-9*-dependent AWA function (*osm-9* rescued only in the CEM + AWC combination) both have highly impaired attraction behavior. This suggests OSM-9 function in AWA is critically important for male attraction behavior, and it is consistent with the published observation that OSM-9 seems to be required for all known functions of AWA [24]. In all cases, *osm-9* rescue in a single class of neurons is not sufficient to fully restore male attraction behavior, indicating that the contributions of each class are to some degree nonredundant; inputs from each class must be combined to achieve full, robust behavior.

A male sensory system without the CEM neurons might be expected to be equivalent to that of a hermaphrodite, but it is not. To clarify, males lacking the CEM neurons, either by L3 ablation or by mutation [*ceh-30(bc272)*], have robust sexual-attraction behavior mediated by AWA and AWC. Hermaphrodites also lack CEM neurons and have AWA and AWC sensory neurons but do not have attraction behavior. Masculinizing the hermaphrodite nervous system demonstrates that the sole determinant of the sex specificity of sexual attraction behavior is the sex of the nervous system. Notably, most masculinized hermaphrodites lack CEM neurons but have robust attraction behavior. Therefore, the core *C. elegans* nervous system must be sexualized: Neurons common to both males and hermaphrodites are subtly modified to serve different sex-specific functions, giving rise to drastic differences in behavior.

Supplemental Data

Supplemental Results, Experimental Procedures, three figures, one table, and one movie are available at http://www.current-biology.com/cgi/content/full/17/21/■■■/DC1/.

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Supplemental Data

The Sensory Circuitry for Sexual Atraction in *C. elegans* Males

Jamie Q. White, Thomas J. Nicholas, Jeff Gritton, Long Truong, Eliott R. Davidson, and Erik M. Jorgensen

Supplemental Results

Hermaphrodite Attraction Pheromones Are Potent and Specific

The pheromones in hermaphrodite-conditioned media are potent. Our standard preparation of conditioned media is made at 10,000 worm-equivaltents/ml (used for all experiments unless otherwise noted), but males respond to conditioned media diluted to 10 worm-equivalents/ml (Figure S2A) and, in some experiments, still respond to dilutions of 1 worm-equivalent/ml (data not shown). Worms enter dauer diapause—an alternative larval stage—under conditions of intense crowding and starvation. Dauer formation is promoted by a secreted pheromone comprised of a mix of several components [S1, S2]. Although dauer pheromone signals the presence of other worms, the attraction pheromone does not appear to be dauer pheromone because conditioned media from *daf-22* mutant hermaphrodites, which fail to secrete dauer pheromone, still elicits a response (Figure S2A). The comparison of male attraction behavior elicited by either wild-type or *daf-22*-conditioned media prepared in parallel reveals no statistically significant differences at four different concentrations (Figure S2A, "p at 95% CI, concentration"). The attraction activity is derived from worms, rather than their bacterial food, because media can be conditioned by adult hermaphrodites in the absence of bacteria (Figure S2B), and media conditioned by bacteria without worms lacks detectable attraction activity (J.Q.W. and L.T., unpublished data). The attraction pheromone appears to be derived specifically from hermaphrodites rather than males. Males respond to male-conditioned media in the two-spot assay, but the response is weaker when compared to hermaphrodite-conditioned media that was prepared in parallel with an equal concentration of animals and tested in parallel on the same days (Figure S2B). Additionally, the analysis of individual males indicates that the response is different compared to hermaphrodite-conditioned media (Movie S1). Males investigate male-conditioned media by slowing, sampling the edge of the source, and repeatedly backing away. In contrast, when responding to hermaphrodite-conditioned media they speed up, circle the source, and sample the source center. The attraction activity can persist in the environment because we can leave conditioned media at 22°C for two weeks with no loss of potency (tested daily, L.T., unpublished data). In summary, hermaphrodites release stable, potent, specific chemosensory cues into their environment that attract males from a distance.

Variation in the Male Sexual-Attraction Response Is Stochastic, Not Developmental

Throughout our analysis, wild-type males failed to show an attraction response in 10%–20% of the positive controls. To understand why wild-type males might fail to exhibit sexual-attraction behavior, we assayed the same individuals in successive experiments. To keep track of individuals, we switched to single-animal assays. To better quantitate behavior in single worm assays, we switched to a numerically scored assay that was amenable to statistical analysis of variance (Supplemental Experimental Procedures). Single animal assays reflected the same trends we observed in assays with three males per plate—wild-type animals exhibited attraction more often than did *osm-9* mutants—but were less robust (Figure S3A). Assays with larger populations might be expected to be even more robust, but are in fact not possible because males encounter one another and become distracted by mechanosensory stimuli, obscuring attraction behavior.

The failure of a single male to respond to hermaphrodite-conditioned media could be stochastic—there is a certain probability that any male will fail to respond to the conditioned media in any given assay. Alternatively, a particular male might fail because of improper wiring during development, and so will always fail. In sets of successive assays on consecutive days, the overall fraction of animals exhibiting attraction behavior was not significantly different from day to day (Figure S3B), but this was not because of the consistent success of particular individuals. Males that failed in one experiment were successful in the next, and vice versa (Figure S3C). This result makes it unlikely that failure of a particular male is due to a developmental defect in that individual. Similarly, *osm-9* mutant males occasionally succeed in finding the conditioned source; when we retest these successful males, they failed. Likewise, *osm-9* males that failed in the first assay succeeded when retested (Figure S3C), so successful attraction in any single *osm-9* male was also stochastic. Thus, it seems likely that residual attraction in *osm-9* is due to a partially functioning sensory system, rather than to developmental differences within the population of animals. In general, these experiments indicate that attraction behavior, like other chemosensory-mediated navigation [S3], is probabilistic at the individual level.

Supplemental Experimental Procedures

Strains

The wild-type is N2 Bristol. All other strains used in this study are shown in Table S1

Hermaphrodite-Conditioned Media

We prepared hermaphrodite-conditioned media by growing standard liquid cultures of hermaphrodites as follows: A population of gravid adults was bleached so that eggs could be collected and starved in S media so that a synchronized population of L1 larvae could be obtained. Cultures were adjusted to 10,000 L1 animals per milliliter and food was added to start synchronous growth. Food was a concentrated (50% v/v) suspension of HB101 bacteria in S media, added to an initial working concentration of 2%. Food was subsequently added as necessary during incubation to prevent

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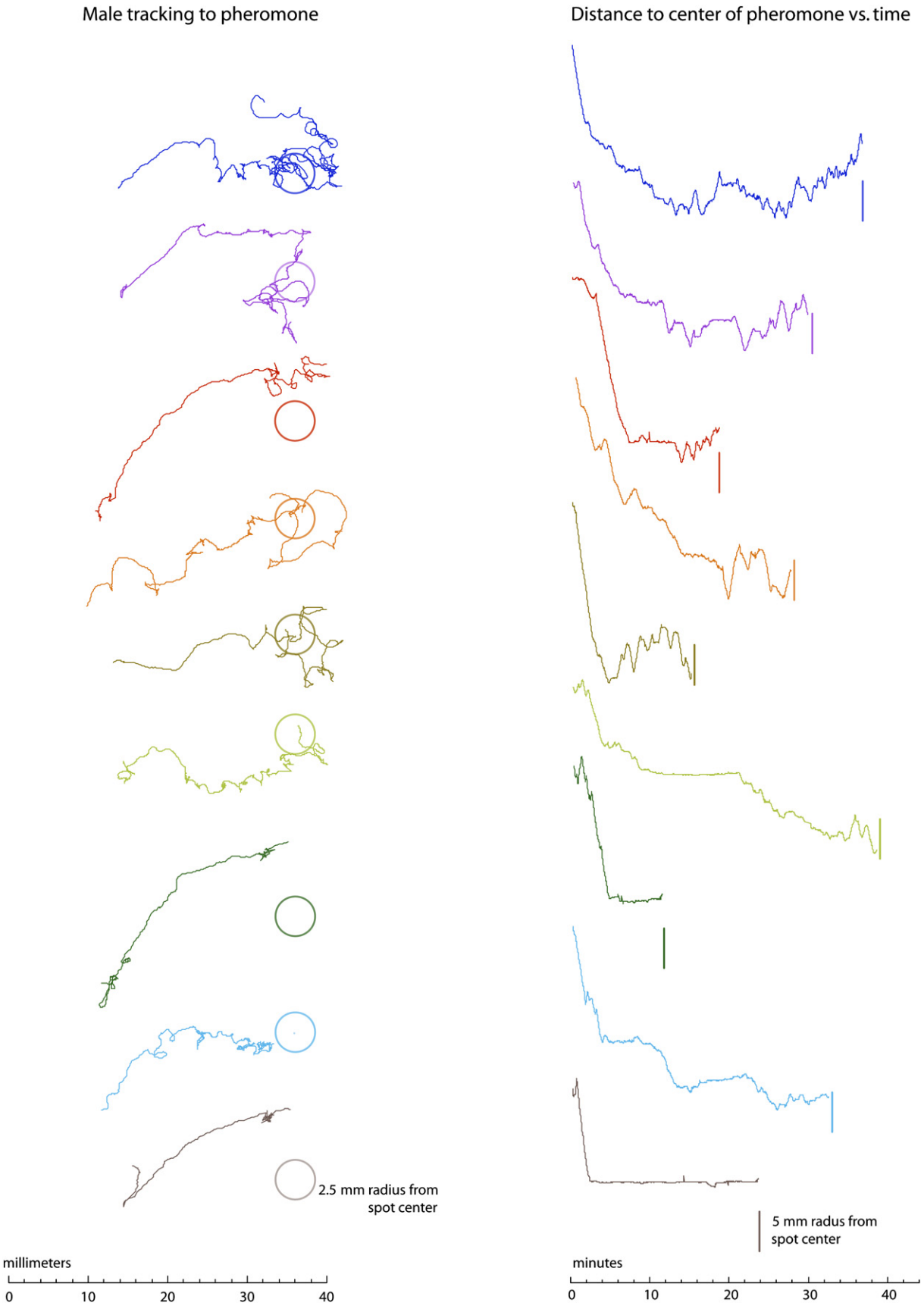

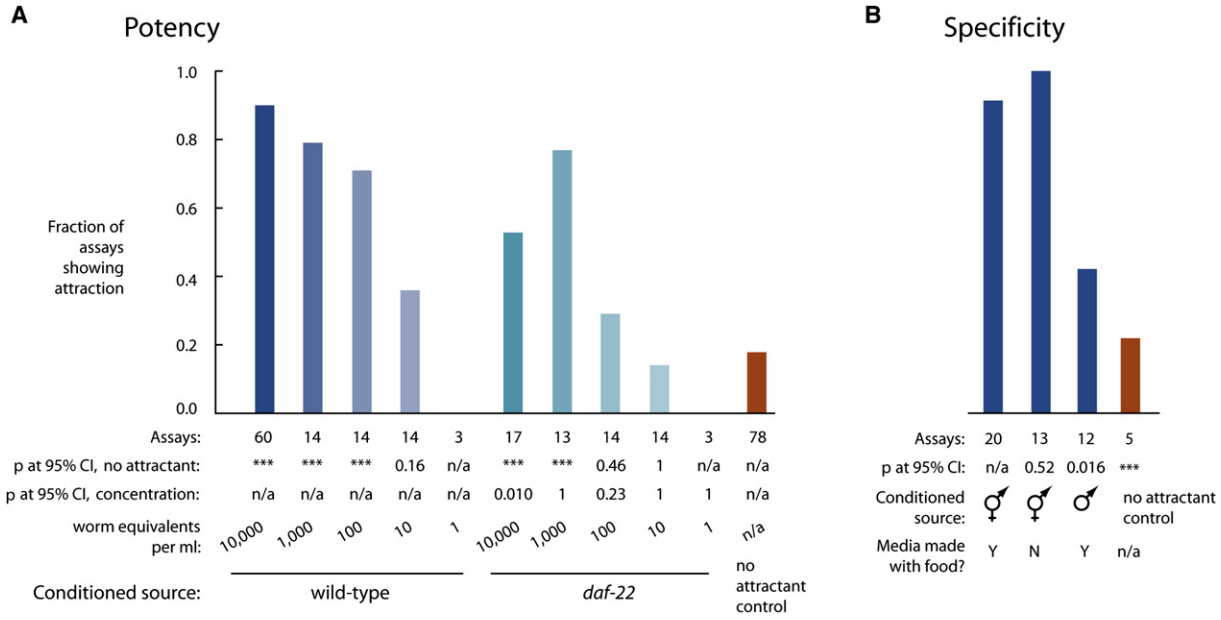

Figure S2. Hermaphrodite Pheromones Are Potent and Specific

(A) The attraction activity is potent and is not dauer pheromone. We assayed the response of males to different dilutions of media conditioned by wild-type and *daf-22* mutant hermaphrodites, which fail to secrete dauer pheromone. Activity was assessed by the indicated number of blind assays. For the experiment shown, the *daf-22*- and wild-type-conditioned media were prepared in parallel and assayed on the same days. Similar results were obtained with three different batches each of *daf-22*- and wild-type-conditioned media with assays performed on multiple days. Undiluted conditioned media is made at 10,000 worm-equivalents/ml. The response to media conditioned by wild-type hermaphrodites is concentration dependent and is lost at dilutions higher than 1:1000 (100 worm-equivalents/ml). The response to media conditioned by *daf-22* hermaphrodites is lost at lower dilutions (below 10 worm-equiv/ml, or 1:100 of original source). Fisher's exact test was used to compare the response at each concentration between *daf*-22 and wild-type conditioned media ("p at 95% CI, concentration") and to compare the response at each concentration with the unconditioned media control ("p at 95% CI, no attractant"). The Bonferroni-Holm correction was applied for multiple comparisons. Exact p values are given in the figure; "***" indicates $p < 0.0001$.

(B) The attraction activity in hermaphrodite-conditioned media is hermaphrodite specific. Media conditioned by hermaphrodites in the absence of bacterial food elicits the same response as media with food. We prepared male-conditioned media by shifting a synchronous culture of *tra-2(ts)* animals to the nonpermissive temperature so that they developed as males, and was compared to hermaphrodite-conditioned media prepared in parallel with an identical concentration of worms (10,000 animals/ml). Media conditioned by *tra-2* males elicits a response, but the response is not robust and is qualitatively different (Movie S1). Fisher's exact test with the Bonferroni-Holm correction for multiple comparisons was used for the comparison of each type of conditioned media to N2 hermaphrodite conditioned media ("p at 95% CI"). Exact p values are given in the figure; "***" indicates $p < 0.0001$.

starvation, usually to a final concentration of 1% on the second day of growth and 2% each day thereafter (the concentration of food added on subsequent days is calculated ignoring the concentration of any food remaining from previous days). It was necessary to supplement the S media used for liquid cultures to at least 25μg/ml of cholesterol to prevent the animals from developing into dauer larvae and arresting. Cultures were grown 3 days at 25°C and harvested when hermaphrodites were young adults with few eggs.

We harvested cultures by removing the worms by gentle centrifugation in a clinical centrifuge for 5 min at 1,000 rpm. No dead worms were observed in the supernatant from this step; the centrifugation is gentle and the worms in the pellet remain healthy and can be used to start another liquid culture. The worm-free supernatant was transferred to a new tube, and the bacteria were removed with a hard centrifugation at 8,000 xg for 60 min in a Sorvall SS-34 rotor. The clarified supernatant was filtered through a 0.2 μm filter and kept frozen in aliquots at −20°C prior to use.

Male-Conditioned Media

Male-conditioned media was prepared from genetically transformed XX animals. CB5362 *tra-2(ar221ts) II; xol-1(y9) X* animals were used for the generation of cultures containing only males. When grown at 15°C, CB5362 animals develop as hermaphrodites or intersex animals containing eggs. When grown at 25°C, CB5362 animals develop as XX males. CB5362 males have normal attraction behavior (Figure 2E) and are fertile and overtly normal for mechanosensory mating behaviors. We grew liquid cultures of CB5362 at 15°C to obtain eggs by bleach and starvation and to generate a synchronized population of L1 larvae. 50% HB101 (v/v) food stock was added to a final concentration of 2% sfor the initiation of growth. Food was added as necessary during incubation so that starvation could be prevented, usually to a final concentration of 1% on the first day of growth and 2% each day thereafter (calculated as above). The cultures were grown at 15°C until the animals reached the late L4 stage and then shifted to 25°C until eggs developed in the adults so that the maternal contribution of TRA-2 could be eliminated [S4]. Eggs were isolated at 25°C by bleaching and a new culture was started at 25°C. The animals in this culture appeared to be exclusively males: We counted 3,000 individual animals and they were all male; if hermaphrodite escapers exist in CB5362 male cultures, they are at a frequency where they would not condition the media (the activity of hermaphrodite-conditioned media is lost below approximately a 1:1,000 dilution, Figure S2). Male cultures were adjusted to 10,000 worms/ml and grown until the males were

Figure S1. Male Chemotaxis to a Spot of Hermaphrodite Pheromone

Nine males were analyzed from a computer tracking experiment. The positions of the males were recorded once per second for 60 min. The left column shows the tracks the males made while they were finding the spot. Circles are centered on the pheromone source and are 5 mm in diameter. The right column shows the distance of the male from the center of the source as time progresses. The bars indicate 5 mm from the source center.

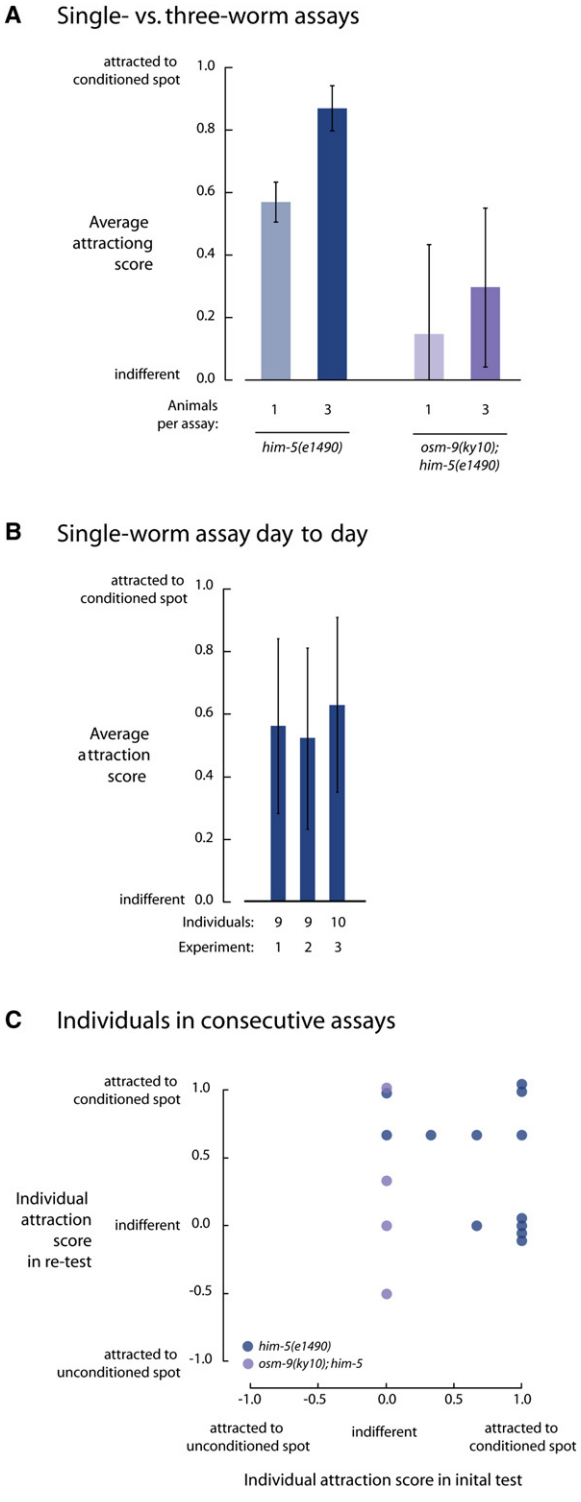

Figure S3. Variation among Individual Responses Is Stochastic, Not Developmental

(A) Single-animal assays can reliably assess attraction behavior but are not directly comparable to three-animal assays. We averaged the attraction response of three sets of ten independent individuals in three consecutive experiments and compared it to the average attraction score of three sets of ten independent three-worm assays on three consecutive days. *him-5* males have a clear attraction response in both assays, but the three-worm assay has a higher attraction score. *osm-9* males have a clearly impaired attraction response in both sets of assays but succeed more often in three-worm assays. Bars represent the average of three experiments; error bars indicate ± 95% confidence interval of the daily mean.

(B) A similar fraction of individual males exhibits attraction behavior in repeated experiments. We assayed three independent sets of at least nine *him-5(e1490)* males on three consecutive days by using the scored attraction assay (Supplemental Experimental Procedures). The same fraction of males showed attraction in each experiment; the means are not significantly different by one-way ANOVA with Bonferroni's multiple comparison test at 95% CI. Error bars indicate ± 95% CI of the mean within each day.

(C) The attraction response of particular individuals is independent from experiment to experiment. We tested the same male in subsequent experiments on consecutive days. In the plot, each individual is represented by a dot. His score in the first experiment is plotted on the ordinate, and his score in the second experiment is the abscissa. There is no relation between the response of an individual in the first experiment and his response in the second, for both *him-5(e1490)* and *osm-9(ky10)* animals (not significant by one-way ANOVA).

1-day-old adults. Male-conditioned media was harvested identically to hermaphrodite-conditioned media.

Tracking Assay: Figures 2A–2D, 6C, and S1

Twenty microliters of conditioned or S complete media were allowed to soak into 50 mm (measured) NGM growth plates supplemented with 0.1% bromophenol blue dye (Sigma-Aldrich ACS reagent, cat. nr. 114391). Spots were allowed to soak in at least 1 hr, and then we spread the plates with 240μl of 2% (v/v in S complete) HB101 bacterial food and allowed them to dry an additional 30 min at room temperature in 10%–20% ambient humidity. Ten animals were released on the plate into a 5μl drop of S complete equidistant from each spot. The assay begins when the release drop dries and the males begin crawling on the plate. Assays were recorded at 1 frame/s with a consumer digital video camera for 2 hr. We processed videos for tracking in ImageJ (http://rsb.info.nih.gov/ij/) by subtracting an image of the plate without males to isolate moving objects and then smoothing and thresholding to produce a movie of binary images. We wrote a tracking plugin for ImageJ that uses the built-in particle analysis feature of the software. The plugin tracks the xy position of the centroid of the worm through the entire movie. If the xy position of a particular worm is within a spot of media, the animal is scored as spending one second of time in that spot. The relative occupancy within a spot (either conditioned or unconditioned) is the total time all worms spend within that spot normalized to the total time in both spots.

Simple Observation Assay: Figures 1, 2, 3, and S2

Plates were prepared identically to the tracking assay except with bromophenol blue in the media rather than the agar to visualize the spots. We blinded each plate both with respect to the genotype of animals on the plate and the identity of the spots (conditioned versus unconditioned). Males were released into a drop of S media as for the tracking assays. Plates were then scored for attraction behavior between 1 to 3 hr. This time window was established by the behavior of wild-type males. Prior to 1 hr, males have not made a convincing track pattern at the conditioned spot. After 3 hr males tend leave the conditioned spot and make tracks elsewhere on the assay plate. Plates that showed attraction behavior appeared similar to the left assay plate in Figure 1; plates that lacked attraction behavior typically appeared similar to the right plate. Males can wander over the unconditioned spot before finding the conditioned spot, so assays with tracks at both spots were considered positive for attraction if one spot had a track density clearly higher than the other. If both spots had approximately equal track patterns, the assay was scored as a failure. At least four positive and four negative control plates were included in each set of blinded plates. Assays were repeated on at least three different days. The total number of plates in each category for all days was combined for contingency analysis with Fisher's exact test. The Bonferroni-Holm correction was applied if multiple pairwise comparisons were made.

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Table S1. Strains Used in This Study

Strain Designation	Genotype	Source or Reference
N2 Bristol	wild-type	Caenorhabditis Genetics Center
EG2717	*him-5(e1490) V*, 8x outcross	Caenorhabditis Genetics Center
EG2718	*him-8(e1489) IV*, 8x outcross	Caenorhabditis Genetics Center
DR476	*daf-22(m130) II*	Cori Bargmann
EG4208	*osm-6(p811) V ; him-8(e1489) IV*	Jorgensen collection
EG3396	*osm-5(p813) X him-5(e1490) V*	Jorgensen collection
EG4209	*osm-3(p802) IV ; him-5(e1490) V*	Jorgensen collection
PS3401	*lov-1(sy582) II ; him-5(e1490) V*	Maureen M. Barr
PT9	*pkd-2(sy606) IV ; him-5(e1490) V*	Maureen M. Barr
PS3398	*lov-1(sy582) II pkd-2(sy606) IV; him-5(e1490) V*	Maureen M. Barr
CB5362	*tra-2(ar221ts) II; xol-1(y9) X*	Caenorhabditis Genetics Center
PR691	*tax-2(p691)*	Jorgensen collection
PR678	*tax-4(p678)*	Jorgensen collection
JT195	*daf-11(sa195)*	Jim Thomas
DR47	*daf-11(m47)*	Jorgensen collection
EG3395	*osm-9(ky10) IV ; him-5(e1490) V*	This study
EG4172	*osm-9(n1601) IV ; him-5(e1490) V*	This study
EG4173	*osm-9(n1603) IV ; him-5(e1490) V*	This study
EG179	*osm-9(ox179) IV*	Jorgensen collection
EG3924	*osm-9(ky10) IV ; him-5(e1490) V ; lin-15(n765ts) X*	This study
VM370	*ocr-1(ak46) V*	Villu Maricq
EG2443	*ocr-2(ox170) IV*	Jorgensen collection
VM380	*ocr-2(ak47) IV ; ocr-1(ak46) V*	Villu Maricq
CX4726	*osm-9(ky10) IV ocr-2(ak47) IV ; ocr-1(ok132) V*	Cori Bargmann
EG4767	*tax-4(p678) III ; osm-9(ky10) IV ; him-5(e1490) V*	This study
CX2205	*odr-3(n2150)*	Jorgensen collection
BX30	*fat-3(wa22)*	Caenorhabditis Genetics Center
MD549	*him-5(e1490) V ; bcIs9[P(pdk-2)::gfp] X lin-15(n765ts) X*	Barbara Conradt
EG4169	*him-8(e1489) IV; oyIs[P(odr-10)::gfp] V*	This study
EG4168	*him-8(e1489) IV ; oyIs48[P(ceh-36)::gfp, lin-15(+)]*	This study
EG4170	*him-8(e1489) IV ; oyIs48[P(ceh-36)::gfp, lin-15(+)] V ; bcIs9[P(pdk-2)::gfp] X*	This study
MD1400	*him-5(e1490) V; bcIs9 ceh-30(bc272) X*	Barbara Conradt
CX5922	*ceh-36(ky640) X ; kyIs140[str-2::gfp] I*	Cori Bargmann
CX4	*odr-7(ky4)*	Cori Bargmann
EG4701, EG4702, EG4703	*him-8(e1489) IV ; bcIs9[P(pkd-2)::gfp] X ceh-30(bc272) X ceh-36(ky640) X ; oxEx1025[P(pkd-2)::gfp, P(ceh-36)::gfp, P(unc-17)::mCherry]*	This study
EG4704, EG4706, EG4707	*him-8(e1489) IV ; odr-7(ky4) X ceh-36(ky640) X ; oxEx1028[P(odr-10)::gfp, P(ceh-36)::gfp, P(unc-17)::mCherry]*	This study
EG4708, EG4709, EG4710	*him-8(e1489) IV ; bcIs9[P(pkd-2)::gfp] X ceh-30(bc272) X odr-7(ky4) X ; oxEx1037[P(pkd-2)::gfp, P(odr-10)::gfp, P(unc-17)::mCherry]*	This study
EG4671, EG4672, EG4671	*bcIs9[P(pkd-2)::gfp] X ceh-30(bc272) X odr-7(ky4) X ceh-36(ky640) X ; oxEx1023[P(pkd-2)::gfp, P(odr-10)::gfp, P(ceh-36)::gfp, P(unc-17)::mCherry]*	This study
EG4222, EG4223	*him-5(e1490) V ; lin-15(n765ts) X ; oxEx790[rab-3::mCherry::egfp::unc-54, lin-15(+)]*	This study
EG4244, EG4255	*osm-9(ky10) IV ; him-5(e1490) V ; lin-15(n765ts) X ; oxEx792[rab-3::mCherry::egfp::unc-54, lin-15(+)]*	This study
EG4312, EG4313, EG4314	*osm-9(ky10) IV ; him-5(e1490) V ; lin-15(n765ts) X ; oxEx826[P(pkd-2)::osm-9(wt)::gfp(S65C)::unc-54, lin-15(+)]*	This study
EG4411, EG4412	*osm-9(ky10) IV ; him-5(e1490) V ; lin-15(n765ts) X ; oxEx882[P(odr-10)::osm-9(wt)::gfp(S65C)::unc-54, lin-15(+)]*	This study
EG4777, EG4778, EG4779	*osm-9(ky10) IV ; him-5(e1490) V ; lin-15(n765ts) X ; oxEx1074[P(odr-10)::osm-9(wt)::mCherry(worm)::unc-54, lin-15(+)]*	This study
EG4408, EG4409, EG4410	*osm-9(ky10) IV ; him-5(e1490) V ; lin-15(n765ts) X ; oxEx881[P(ceh-36)::osm-9(wt)::gfp(S65C)::unc-54, lin-15(+)]*	This study
EG4417, EG4418, EG4419	*osm-9(ky10) IV ; him-5(e1490) V ; lin-15(n765ts) X ; oxEx884[P(odr-10)::osm-9(wt)::mCherry(worm)::unc-54, P(pkd-2)::gfp(S65C), lin-15(+)]*	This study
EG4424, EG4426, EG4427	*osm-9(ky10) IV ; him-5(e1490) V ; lin-15(n765ts) X ; oxEx894[P(odr-10)::osm-9(wt)::mCherry(worm)::unc-54, P(ceh-36)::osm-9(wt)::gfp(S65C)::unc-54, lin-15(+)]*	This study
EG4428, EG4429, EG4432	*osm-9(ky10) IV ; him-5(e1490) V ; lin-15(n765ts) X ; oxEx899[P(ceh-36)::osm-9(wt)::gfp(S65C)::unc-54, P(pkd-2)::osm-9(wt)::gfp(S65C)::unc-54, lin-15(+)]*	This study
EG4401, EG4403, EG4404, EG4405	*osm-9(ky10) IV ; him-5(e1490) V ; lin-15(n765ts) X ; oxEx876[P(odr-10)::osm-9(wt)::mCherry(worm)::unc-54, P(ceh-36)::osm-9(wt)::gfp(S65C)::unc-54], P(pkd-2)::osm-9(wt)::gfp(S65C)::unc-54, lin-15(+)]*	This study
EG4396, EG4397, EG4398	*osm-9(ky10) IV ; him-5(e1490) V ; lin-15(n765ts) X ; oxEx866[P(rab-3)::osm-9(wt)::mCherry(worm)::unc-54, lin-15(+)]*	This study
EG4386, EG4389, EG4392	*him-5(e1490) V ; lin-15(n765ts) X ; oxEx863[P(rab-3)::fem-3(wt)::mCherry(worm)::unc-54, pkd-2::gfp(S65C), lin-15(+)]*	This study

Numerically Scored Observation Assay: Figures 4D–4F, 5, and 6B

Numerically scored observation assays account for the observation that males occasionally behave as if they prefer one spot over the other on plates with two equivalent, unconditioned spots, and that mutant and ablated animals occasionally seem to prefer the unconditioned spot. That is, a scored observation assay discriminates between the left and right spots, even when those spots are equivalent. Assays were prepared and blinded identically to those for the simple observation assay; the only difference is manner in which the assays scored. We visually scored the track pattern and position of the males after 1, 2, and 3 hr. After unblinding, if the male made a track pattern that went to the conditioned spot, or if the male was at the conditioned spot at that time point, the plate was given a score of +1. If the male made a track pattern that was indifferent to the spots, the plate was given a score of 0. If the male made a track pattern that went to the unconditioned spot, or if the male was at the unconditioned spot at that time point, the plate was given a score of −1. If there were tracks that correlated with both spots, the score was based on the spot where the track density was highest. Usually, this was easily discernable. The scores for three time points were added and normalized. The score ranges from −1 to +1; −1 indicates that the male preferred the unconditioned spot, 0 indicates the male was indifferent to the spots, and +1 indicates that the male preferred the conditioned spot. To derive an average score for a strain or condition, we performed three independent sets of four to six assays on separate days and averaged the scores, weighted by the number of assays performed on each day. Positive and negative control plates were always included, typically four to six of each. One-way ANOVA was used to compare differences between strains. The scores of all assays for each valid day of experiments (both positive and negative controls responded as expected) were considered as a group; for comparisons to a single control group, we used Dunnett's multiple comparison test. For comparisons of all strains with one another, we used Bonferroni's correction for multiple comparisons.

Single-Worm Assays: Figures 4B and S3

These assays are numerically scored and use one male instead of three. Statistical analysis was the same as that for scored observation assays.

Laser Ablations: Figures 4B and 4C

Ablations were performed according to standard protocols. Worms were mounted on 1.5% agarose pads (w/v in M9 buffer) in a drop of 25 mM NaN_3 (in M9). CEM ablations were performed in a strain containing an insertion of a *pkd-2::gfp* transcriptional fusion, in which the male-specific neurons express GFP in adults. The *pkd-2* promoter does not express GFP in L3 or L4 larvae, so the CEM neurons were identified by anatomical position and successful ablation scored in the adult after the behavioral assay had been completed. The AWA, AWC, and ASE neurons were identified by specific GFP expression (see strains below). Animals were allowed to recover from ablations on an NGM plate with HB101 food. To keep postoperative males from committing suicide by crawling up the side of the dish, we clipped 18 gauge copper wire around the edge of the agar. After behavioral assays, males were examined at high magnification so that the success of ablation could be judged. Successfully ablated animals had no damage visible by differential interference contrast (DIC), had nearby neurons intact by DIC, and lacked the relevant GFP-labeled neurons.

The transgene insertions driving GFP expression in AWA and AWC are on the same chromosome, and we were unable to make the double-transgenic marked strain. Thus, for the CEM, AWC, AWA triple ablations, both CEM and AWA neurons were identified by anatomic position, and AWC was identified by GFP expression. To judge the success of anatomic identification of AWA, we identified it first by DIC microscopy in a strain in which AWA was marked with GFP and then verified proper identification by visualizing fluorescence. In one experiment, 40/42 AWA neurons were correctly identified by anatomy.

For each ablated animal we assayed at least one mock-treated animal. One-way ANOVA with Dunnett's multiple comparison test was used to compare the mock and ablated animals separately for L3 and L4 ablations.

The following strains were used for laser ablation: for CEM ablations, MD549 *him-5(e1490) V ; bcIs9[P(pdk-2)::gfp] X lin-15(n765ts) X*, generously provided by Barbara Conradt; for AWA ablations, EG4169 *him-8(e1489) IV; oyIs[P(odr-10)::gfp] V*; for AWC and ASE ablations, EG4168 *him-8(e1489) IV; oyIs48[P(ceh-36)::gfp, lin-15(+)] V*; and for CEM, AWA, AWC triple ablations, EG4170 *him-8(e1489) IV; oyIs48[P(ceh-36)::gfp, lin-15(+)] V; bcIs9[P(pdk-2)::gfp] X*. Parent strains containing *oyIs48* and *oyIsP(odr-10::gfp)* were generously provided by Piali Sengupta.

Attraction Behavior of Cell-Fate Mutants: Figures 4D–4F

We generated mutant strains carrying different combinations of *ceh-30(bc272)*, *odr-7(ky4)* and *ceh-36(ky640)*. In *ceh-30(bc272)* mutants, the CEM neurons fail to survive programmed cell death in males (Barbara Conradt and Phillip Grote, personal communication). In *odr-7(ky4)* mutants, all known characteristics of differentiated AWA fate are lost [S5]. In *ceh-36(ky640)* mutants, all known characteristics of differentiated AWC fate are lost [S6]. These three mutations are all on the X chromosome, so the recombinant double- and triple-mutant lines were identified by quantitative polymerase chain reaction (PCR) and verified by sequencing. All three mutations are not completely penetrant (J.Q.W., unpublished data, and [S5]), so to verify the absence of AWA, AWC, and the CEM neurons, we generated mutant lines containing transgenes that express GFP specifically in the AWA, AWC, and the CEM neurons and neuronal mCherry as an array marker (Table S1). The arrays were functional because frequently (30%–50% of individuals, depending on the line) the GFP marker was expressed inappropriately, meaning that a properly differentiated neuron was present when it should not have been. For each line, we assayed individual mutant males, recovered the animal, and mounted him for microscopy. We only included an animal in our analysis if he had the array (neuronal mCherry) but did not inappropriately express GFP. Animals containing this array and also expressing GFP in sensory neurons must possess at least one properly differentiated AWA, AWC, or CEM sensory neuron, and the results of these assays were discarded before the data were unblinded.

Neuron-Specific Rescue of *osm-9*: Figure 5

We expressed the wild-type *osm-9* cDNA in specific neurons in an *osm-9(ky10) IV ; him-5(e1490) V ; lin-15(n765ts) X* background (EG3395, Table S1) by using cell-type-specific promoters. Expression constructs were generated with the Multisite Gateway system (Invitrogen). We generated a set of promoterome-compatible, 4-1 Entry vectors to drive expression in AWA, AWC and ASE, the male-specific neurons, and the entire nervous system. The promoters used were *odr-10*, *ceh-36, pkd-2*, and *rab-3*, respectively. The *osm-9(wt)* cDNA was cloned into a 1-2 Entry vector upstream of either GFP or mCherry in an artificial operon, a design based on a similar system by Coates and de Bono [S7]. All expression constructs used a generic *unc-54* 3′ untranslated region (UTR) in the Multisite 2-3 position. So that the final expression constructs could be generated, Entry vectors were recombined in a Multisite LR reaction with (1) the promoter of choice, (2) *osm-9(wt)::operon::gfp/mCherry*, (3) *unc-54* 3′ UTR, and (4) pDEST-R4-R3. Expression constructs were injected singly or mixed in the same array for combination rescue; injection concentrations were empirically determined and were generally in the range of 20–60ng/μl per expression construct. *lin-15(+)* was used as a coinjection marker at 5–10ng/μl. At least six lines were generated for each combination, and males from each line were assayed for male attraction in at least six numerically scored, blind assays per day on three separate days for a total of at least 18 assays per line. The scores for the lines with the top three average scores over the three separate days were combined for Figure 5. One-way ANOVA with Dunnett's correction for multiple comparisons was used to compare each line with transgenic control lines (wild-type controls, EG4222 and EG4223; *osm-9* mutant controls, EG4244 and 4255; see Table S1).

Masculinizing the Hermaphrodite Nervous System: Figure 6

We used the Multisite Gateway system to generate an expression vector with *fem-3* expressed by the *rab-3* promoter: We cloned the *fem-3* cDNA into our artifical-operon 1-2 Entry vector in place

of *osm-9* (above) and then generated a final expression construct with our *rab-3* 4-1 Entry and *unc-54* 3′ UTR 2-3 Entry, denoted P(*rab-3*)::*fem-3*::*mCherry*. We injected this expression vector at 40–80ng/μl together with a P(*pkd-2*)::*gfp* reporter at 10ng/μl and l*in-15(+)* as a coinjection marker at 5–10ng/μl. We used blind, numerically scored assays to assay transgenic XO and XX animals from 12 independent lines on three separate days, six assays per line, in parallel with six assays each of transgenic control hermaphrodites and males (EG4222 and EG4223, identical array except with *gfp* in place of *fem-3*, see Table S1). Results in Figure 6 are from the three lines with the highest average attraction scores over the 3 days. We used one-way ANOVA with the Bonferroni correction for multiple comparisons to compare each line with every other.

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