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Report

Neural Sex Modifies the Function of a *C. elegans* Sensory Circuit

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Summary

Though sex differences in animal behavior are ubiquitous, their neural and genetic underpinnings remain poorly understood. In particular, the role of functional differences in the neural circuitry that is shared by both sexes has not been extensively investigated. We have addressed these issues with *C. elegans* olfaction, a simple innate behavior mediated by sexually isomorphic neurons. Though males respond to the same olfactory attractants as do hermaphrodites, we find that each sex has a characteristic repertoire of olfactory preferences. These are not secondary to other sex-specific behaviors and do not require signaling from the gonad. Sex-specific olfactory preferences are controlled by *tra-1*, the master regulator of *C. elegans* sexual differentiation. Moreover, the genetic masculinization of neurons in an otherwise wild-type hermaphrodite is sufficient to switch the sexual phenotype of olfactory preference behavior. These studies reveal novel and unexpected sex differences in a *C. elegans* sensory behavior that is exhibited by both sexes. Our results indicate that these differences are a function of the chromosomally determined sexual identity of shared neural circuitry.

Results

C. elegans Olfaction Is Sexually Dimorphic

Behavioral differences are an integral part of animal sexual dimorphism. In vertebrates, gonadal steroids have long been known to have critical roles in regulating sex differences in behavior [1]; more recently, gonad-independent events regulated by chromosomal sex have been implicated in these processes [2, 3]. In *Drosophila*, a single gene, *fruitless* (*fru*), has been shown to control male-specific behaviors by specifying masculine properties in multiple types of fly neurons, suggesting that *fru* might be a master regulator of male behavioral circuitry [4–7]. However, the relative complexity of these systems has made it difficult to chart the connection between chromosomal sex and the effectors of sex differences in neural structure and function.

The simple and exceptionally well-characterized nervous system of *C. elegans* [8] makes it an ideal model in which to address these issues. This species has two sexes: XX hermaphrodites (essentially somatic females that can produce some sperm for use in self fertilization) and X0 males. Sexual identity is determined in each somatic cell through a genetic pathway that converges on the master switch *tra-1* [9]. The *C. elegans* nervous system harbors two distinct types of sexual dimorphism [10]. First, each sex possesses a unique set of neurons that control sex-specific behaviors, e.g., hermaphrodite egg laying and male mating. Second, more subtle sex differences exist in the *C. elegans* "core nervous system," the 294 neurons shared by both sexes [8]. These differences are apparent at the ultrastructrual level [11] and as differences in gene expression: Both *srd-1* and *srj-54* are male-specifically expressed in neurons common to both sexes [10, 12]. Though their functional significance is unknown, these differences in the core nervous system indicate that some sex differences in behavior could result not from differences in gross neuroanatomy but from the functional modulation of common circuitry.

To ask whether sex differences in the core nervous system might modulate the expression of behaviors common to both sexes, we examined olfaction, a sensory behavior mediated by a small number of non-sex-specific sensory neurons and interneurons [13, 14]. We compared the olfactory responses of adults of each sex to four previously characterized volatile attractants [15]. We found that males showed attractive responses to all four odorants at nearly all dilutions tested (Figure 1), indicating that male olfactory responses are qualitatively similar to those of hermaphrodites [15]. However, in many cases, the strength of the male response was significantly lower, such that fewer animals migrated to the odorant spot. In particular, for diacetyl (da) and benzaldehyde (bz), we observed a statistically significant reduction in the male chemotaxis index (CI) compared to that of hermaphrodites in all but one case. In contrast, male responses to pyrazine (py) and butanone (bu) were generally comparable to those of hermaphrodites, with statistically significant differences observed only at 1:100 dilutions. These results suggest that *C. elegans* exhibits odorant- and concentration-specific sex differences in olfaction.

To test this possibility more directly, we developed a more specific test of olfactory function. In this olfactory-preference assay, animals are simultaneously presented with two attractive odorants originating from opposite sides of the assay plate (Figure 2A). If male olfactory function is simply less efficient than that of hermaphrodites, males would be expected to distribute themselves among the two odorant spots in the same relative number that hermaphrodites do. However, if there are more specific sex differences in olfaction, males and hermaphrodites might exhibit differences in their relative affinity for the two odorants. We quantified the outcomes of this assay with the olfactory preference index (OPI), which varies from −1 (indicating a complete preference for odorant A) to +1 (a complete preference

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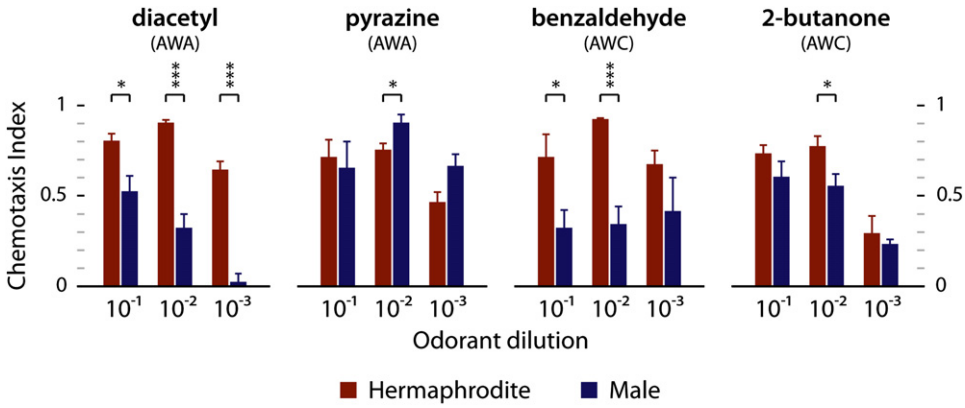

Figure 1. Hermaphrodites and Males Have Different Olfactory Responses

Young adult hermaphrodites (red) and males (blue) were assayed in sex-segregated populations in single-odorant olfaction assays [15]. Three dilutions of the odorants da and py (sensed in hermaphrodites by AWA) and bz and bu (sensed in hermaphrodites by AWC) were tested. Each data point represents the weighted mean of 4 to 12 assays each containing ~50 animals. Error bars show the weighted SEM. The statistical significance of sex differences in CI was determined with a Student's t test. In all figures, statistical significance is indicated with asterisks as follows: "***" indicates $p < 0.001$, "**" indicates $p < 0.01$, and "*" indicates $p < 0.05$.

for odorant B). An OPI of 0 indicates that animals migrate to each of the two spots equally.

With this assay, we tested the responses of animals to four different pairs of attractants (Figure 2B). Interestingly, we found significant sex differences in olfactory preference in all four of these assays. This was true for pairs of attractants sensed in hermaphrodites by two different sensory neuron types (da-bz and py-bz) as well as those sensed by the same neuron (da-py and bu-bz). The most dramatic difference was seen in the da-py assay, in which both odorants are sensed in hermaphrodites by the AWA neurons. Although most hermaphrodites migrated to da ($OPI^{H}_{da\text{-}py} = -0.48 \pm 0.05$), most males preferred py ($OPI^{M}_{da\text{-}py} = 0.59 \pm 0.05$). A model in which male olfactory responses are simply less efficient than those of hermaphrodites cannot easily account for these results; instead, these findings demonstrate that the nature of *C. elegans* olfactory behavior itself differs between the sexes.

Sex Differences in Olfaction Do Not Depend on Other Sex-Specific Behaviors, the CEM Neurons, or the Gonad

These differences in olfactory behavior could reflect alterations in olfaction itself. Alternatively, it is possible that these differences are secondary consequences of other sexually dimorphic behaviors. To test this possibility, we first assayed the olfactory preferences of isolated individuals. Because these animals will not encounter potential mates, males will initiate the mating

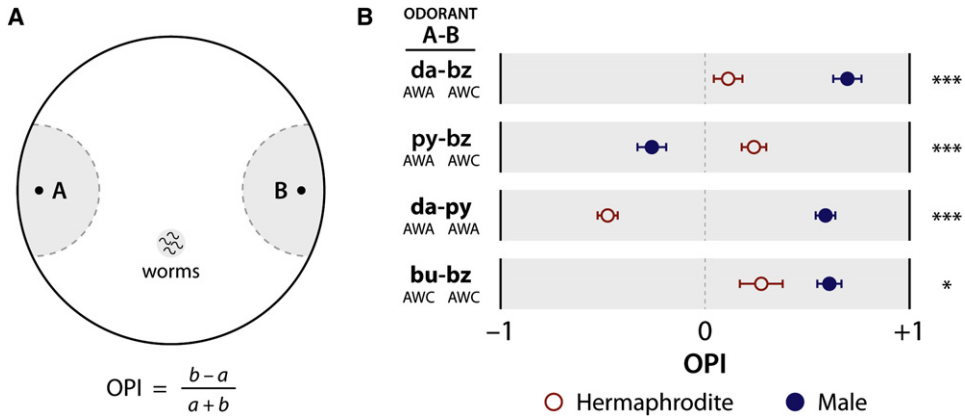

Figure 2. Each Sex Has a Specific Set of Olfactory Preferences

(A) The olfactory-preference assay is a modification of the single-odorant assay in which the control spot ("A") is replaced with a second attractive odorant. A sex-segregated population of worms is placed 1 cm below the center of the plate. After 45 min, the number of animals within 2 cm of each spot is counted and used to calculate the olfactory preference index (OPI). This assay minimizes any potential confounding effects of sex differences in activity (e.g., movement rate or mating drive) or sensitivity to olfactory attractants because it measures the relative difference in attraction to two different odorants.

(B) The OPI of adult hermaphrodites (open red circles) and males (closed blue circles) is shown for each of the four odorant pairs indicated at the left. Each point represents the weighted mean of at least ten olfactory-preference assays, each containing ~50 animals. Error bars indicate weighted SEM. The significance of sex differences in OPI for each odorant pair was determined with a Student's t test.

program far less frequently. We found that the assaying animals in isolation had no effect on olfactory preference (Figure S1 in the Supplemental Data available online). We also examined the behavior of animals in mixed-sex populations, in which the presence of hermaphrodites should suppress the male mate-searching drive [16]. Again, we found that sex differences in olfactory preference remained clearly apparent (Figure S1), showing that male mating behavior, the mate-searching drive, and male-hermaphrodite interactions do not significantly alter sex-specific olfactory preference.

In the head, the only known sex difference in *C. elegans* sensory anatomy is the male-specific CEM sensory neuron class. These four cells have recently been found to be important for male responses to secreted cues produced by *C. elegans* hermaphrodites [17] and by *C. remanei* females [18]. To ask whether the CEM neurons might contribute to sex differences in olfaction, we examined *ceh-30(n4289)* mutant males, in which the CEMs undergo embryonic programmed cell death, just as they do in wild-type hermaphrodites [19]. In the da-py olfactory preference assay, the behavior of *ceh-30* males was indistinguishable from that of wild-type males (Figure S1), indicating that the CEMs are not required, at least in this assay, for male-specific olfaction.

Signals from the gonad have also been implicated in male-specific behavior in *C. elegans* [16]. To ask whether the gonad regulates olfactory preference, we removed its precursors by laser ablation. When either the entire gonad primordium (the Z1–Z4 cells) or germline precursors alone (Z2 and Z3) were ablated, males and hermaphrodites continued to display robust sex differences in da-py olfactory preference, showing no significant difference from their respective mock-ablated controls (Figure 3). Thus, neither the germline nor the somatic gonad provides signals necessary for sex differences in olfactory preference.

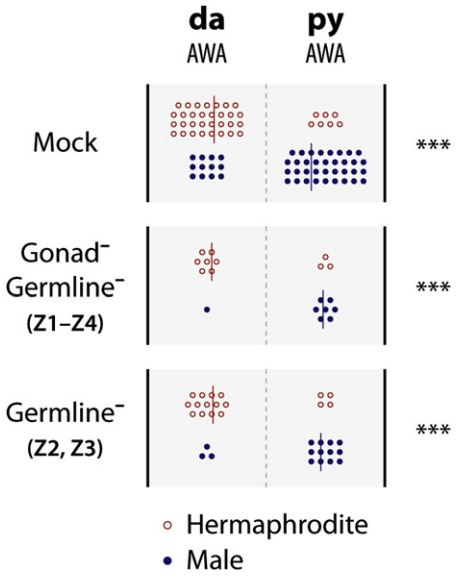

Figure 3. Sex Differences in Olfactory Preference Do Not Require the Gonad

Mock-ablated, germline- and gonad- (Z1–Z4) ablated, and germline- (Z2, Z3) ablated animals were tested individually for da-py olfactory preference behavior as young adults. Behavioral responses were determined with a modified olfactory-preference assay as described in the Experimental Procedures. Each data point represents the odorant-preference behavior of single animal (open red circle, hermaphrodite; closed blue circle, male). Vertical bars indicate the median response of each group of animals. Logistic regression was used for the determination of the statistical significance of the sex difference in behavior in each of the three groups.

Olfactory Preference Is a Property of Neural Sex

Essentially all somatic sexual dimorphisms in *C. elegans* are controlled by the master-switch gene *tra-1* [9], such that XX animals lacking *tra-1* function are transformed to fertile pseudomales [20]. Consistent with this, we found that *tra-1* loss resulted in a complete sex reversal of olfactory preference (Figure 4A): In both the py-bz and da-py assays, the behavior of XX pseudomales was statistically indistinguishable from that of wild-type X0 males. *tra-1* XX pseudomales also exhibited male-like responses in single-odorant assays (Figure S2). Thus, like other somatic sexual characteristics, sex differences in olfactory preference appear to be completely specified by *tra-1.*

If sex-specific olfactory preference is mediated by neural sex, then it should be possible to change the sexual phenotype of behavior by specifically switching the sexual identity of the nervous system. The overexpression of FEM-3, a ubiquitin-ligase cofactor that promotes the degradation of TRA-1A in males [21], is sufficient to masculinize XX animals [22]. Thus, the overexpression of *fem-3(+)* under the control of a pan-neural promoter (P*rab-3*) should specifically masculinize the nervous system but have no effect on the rest of the body [17]. We found that hermaphrodites carrying the P*rab-3::fem-3(+)* transgene *oxEx862* had a normal hermaphrodite body morphology but were sex transformed in the core nervous system, by using the male-specific expression of *srj-54::YFP* as a marker for neural sex (Figure S3). In contrast, these animals did not harbor significant numbers of male-specific neurons, presumably because the neural P*rab-3* driver is not expressed sufficiently early to switch the sex of sexually dimorphic cell lineages that generate these neurons [10].

We found that the olfactory preference behavior of hermaphrodites carrying P*rab-3::fem-3(+)* was completely masculinized: Unlike wild-type hermaphrodites, these animals strongly preferred py in the da-py context (Figure 4B). These results indicate that the sexual phenotype of olfactory preference is a property of the sexual identity of the core nervous system. To define the neural focus through which sexual identity regulates olfaction, we tested animals in which specific subsets of the nervous system were sex reversed. We found that the masculinization of hermaphrodite sensory neurons with P*osm-5::fem-3(+)* [23, 24] resulted in male-like olfactory preference, whereas the masculinization of a large set of interneurons and motor neurons with P*glr-1::fem-3(+)* [25] had no apparent effect (Figure 4B). We conclude that olfactory-preference behavior is determined by the chromosomal sex of the worm's sensory apparatus.

Discussion

How Does Sex Modify Olfactory Function?

Our results indicate that the sexual identity of shared sensory neurons mediates sex differences in olfactory

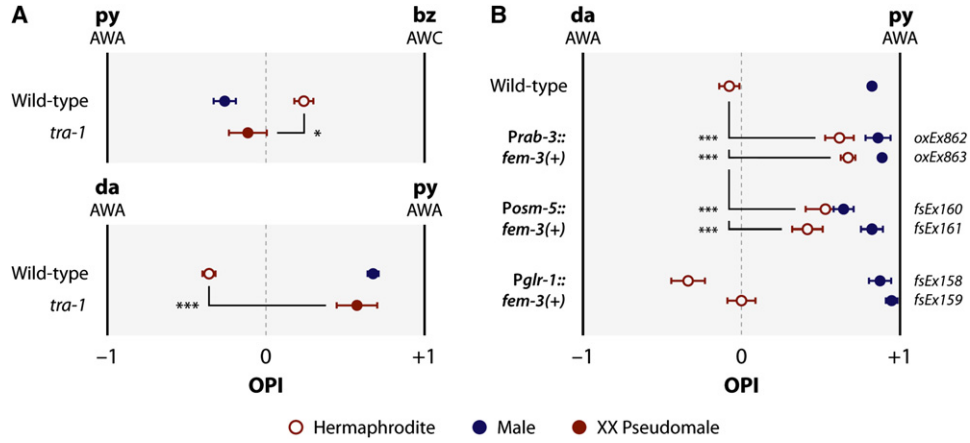

Figure 4. Sex Differences in Olfactory Preference Are Determined by the Sexual Identity of the Nervous System

(A) *tra-1(e1099)* XX pseudomales were assayed for py-bz and da-py olfactory preference. In both cases, the loss of *tra-1* function led to the complete masculinization of the behavior of XX animals.

(B) Animals carrying three different *fem-3(+)* overexpression transgenes were assayed in the da-py olfactory-preference assay. Two different transgenic lines were tested for each construct. P*rab-3::fem-3(+)* (*oxEx862* and *oxEx863*) expresses *fem-3(+)* throughout the nervous system, P*osm-5::fem-3(+)* (*fsEx160* and *fsEx161*) expresses *fem-3(+)* in all sensory neurons [23, 24], and P*glr-1::fem-3(+)* (*fsEx158* and *fsEx159*) expresses *fem-3(+)* in a large set of interneurons and motor neurons [25]. The wild-type averages are derived only from those assays that were carried out in parallel with assays on transgenic strains. Each point represents the weighted mean and standard error of at least six assays with an average of 56 animals per assay (wild-type), 25 animals per assay [P*rab-3::fem-3(+)* and P*osm-5::fem-3(+)*], or 38 animals per assay [P*glr-1::fem-3(+)*]. The statistical significance was determined by ANOVA with Bonferroni post-hoc tests.

Error bars indicate the weighted SEM.

function. There are several possibilities by which this could occur, including the differential expression of odorant receptors, differences in signal transduction within sensory neurons, differences in presynaptic aspects of connectivity and neurotransmission, and differences in neural sensitivity to neuropeptides or other neuromodulators. It is formally possible that odorant responses are mediated by different sets of sensory neurons in males than in hermaphrodites, though this seems unlikely. We have examined the expression of the diacetyl receptor gene *odr-10* in males and detected no sex difference (K.H.L., unpublished data); however, this result must be interpreted cautiously because subtle differences might not be apparent from reporter-gene expression. At the level of connectivity, no obvious sex differences in the anatomy of the *C. elegans* olfactory circuit have been described. However, because synaptic connections in the male head have not yet been reconstructed by ultrastructural analysis, subtle but critical sex differences in wiring might exist. Interestingly, our results indicate that olfactory preference cannot always be predicted from animals' responses to single odorants alone, especially in males, suggesting that differences in sensory integration might contribute to sex-specific olfactory preference. In addition, our results do not rule out the possibility that the sex-specific nervous system in males or hermaphrodites might also have a role in influencing olfaction. Distinguishing between these possible mechanisms will require a more precise understanding of the neural foci of the sex-determination pathway in modulating olfaction.

Why might *C. elegans* chemosensory behaviors differ by sex? At least two differences in the biology of the sexes might be relevant. First, hermaphrodites and males differ in their sensory requirements for reproduction. Although hermaphrodites are self fertile and do not require a mate to reproduce, males enjoy no such luxury. Males appear to locate hermaphrodites at least in part through chemosensory cues [16–18, 26]. Thus, some of the differences in sensory behavior between the sexes could be secondary to the male's need to find appropriate mating partners. Second, it is quite likely that the two sexes have different nutritional needs. Hermaphrodites must generate large volumes of cytoplasm for oocyte production; males might require more energy to support a higher level of exploratory activity. Different sensory preferences might allow animals to locate nutrition sources most commensurate with these physiological demands. Although these two possibilities are not mutually exclusive, we favor the second because it seems less likely that the sex-specific ability of animals to respond to sex pheromones would give rise to obligate differences in olfactory preference in all four of the odorant combinations we tested.

The Regulation of Behavior by Neural Sex

Our results demonstrate that the *C. elegans* core nervous system is cell-autonomously "tuned" by genetic sexual identity to regulate a behavior fundamental to both sexes. Interestingly, a series of recent results in vertebrate systems has indicated that some sex-specific neural characteristics can be generated by cell-autonomous mechanisms [2, 3]. This suggests that a primary sex-determination pathway—i.e., a pathway triggered by the primary sex-determining cue—acts not only in the vertebrate gonad but also in the central nervous system itself. Our findings, together with recent work in *Drosophila* [4–7], indicate that invertebrate models provide a unique opportunity to characterize the mechanisms of neural-sex determination and differentiation. Moreover, sex differences present a powerful means

for understanding how the properties of shared neural circuitry can be modulated to bring about discrete changes in behavior.

Experimental Procedures

Nematode Genetics and Transgenes

C. elegans cultures were grown on nematode growth medium (NGM) plates seeded with *E. coli* OP50 as described [27]. The following mutant alleles were used: *tra-1(e1099)* III, *pha-1(e2123ts)* III, *him-5(e1490)* V, *lin-15(n765)* X, and *ceh-30(n4289)* X. *tra-1* XX pseudomales were obtained from the self progeny of *tra-1(e1099)/pha-1(e2123)* hermaphrodites. Except in the *tra-1* experiments, all strains contained the *him-5(e1490)* mutation, which increases the frequency of spontaneous males in self-fertilizing populations to ~30%–35%.

Strains containing the P*rab-3::fem-3(+)::mCherry* transgenes *oxEx862* and *oxEx863* were generously provided by J. White and E. Jorgensen [17]. To make P*osm-5::fem-3(+)::mCherry* (*fsEx160* and *fsEx161*) and P*glr-1::fem-3(+)::mCherry* (*fsEx158* and *fsEx159*), we polymerase chain reaction (PCR) amplified the *osm-5* [23, 24] and *glr-1* [28] promoters and made 4-1 Entry clones for use in the Multisite Gateway System as described [17]. Extrachromosomal arrays were generated by the coinjection of the *fem-3(+)* construct at 50–75 ng/μl with a *coelomocyte::GFP* marker. The expression pattern of each transgene was verified by the observation of fluorescence from mCherry, encoded by the distal open reading frame (ORF) in these operon-based constructs. In behavioral experiments with these transgenes, only those animals showing clear mCherry expression (or, in the case of *oxEx862* and *oxEx863*, the rescue of the *lin-15* Muv phenotype) were assayed.

The following transgenes were used for the marker-gene expression studies in Figure S3: *fsIs6[srj-54::YFP + cc::GFP]*, *bxIs14[pkd-2::GFP + pBX1]*, *oxEx862[*P*rab-3::fem-3(+)::mCherry + pkd-2::GFP + lin-15(+)]*, and *zdIs13[tph-1::GFP].*

Behavioral Assays

Single-odorant assays were carried out as described previously [15], except that we increased the radius around the odorant spot within which responders were scored to 2 cm. The olfactory-preference assay is a modified version of this procedure in which the control (ethanol) spot is replaced with a second, distinct ethanol-diluted odorant. Olfactory preference behavior is quantified with the olfactory preference index, where OPI = $(b - a)/(a + b)$, with a and b representing the number of animals within a 2 cm radius of odorant spot A or B, respectively, after 45 min.

To remove any possible effect of age variance in olfactory behavior, we used age-synchronized cultures. This was carried out by synchronized egg laying rather than hypochlorite treatment so that any potential side effects of larval starvation could be avoided. In brief, 20 gravid hermaphrodites were allowed to lay eggs on a seeded plate for 2 hr and were then removed. The resulting progeny matured in a relatively synchronous manner. Because P*rab-3::fem-3(+)* and P*osm-5::fem-3(+)* hermaphrodites laid very late-stage eggs (see Figure S3), animals carrying these transgenes were manually staged as mid-L4s. In all cases, animals were sex segregated as L4 larvae and transferred to single-sex holding plates overnight before behavioral assays.

Laser Ablation

We followed a standard protocol to ablate gonad precursor cells [29]. Ablations were carried out in young L1 larvae; the success of the operation was confirmed by the differential interference contrast (DIC) examination of adults after the behavioral assay was carried out. Any animals in which the gonad or germline did not appear to be completely absent were censored. Laser-operated (or mock-operated) animals were recovered, segregated by sex at L4, and individually tested as young adults in a single olfactory-preference assay. Animals were scored as responding to a particular odorant if, after 30 min on the assay plate, their distance from that odorant source was less than 40% of the distance to the other odorant source. (This constraint traces an arc around each odorant source, the radius of which varies from ~2.5 cm at the plate's equator to ~2.8 cm at its edge.)

Statistical Analyses

For chemotaxis and olfactory preference assays, weighted means and standard errors of the mean (SEMs) were calculated with Stata 9 (StataCorp LP [College Station, TX]). We used the total number of worms in each single-odorant assay or the number of responders in each olfactory preference assay to weigh the mean and SEM. We examined sex differences in olfaction in wild-type animals with a two-sample Student's t test assuming equal variances. Comparisons of the behavior of wild-type and mutant or transgenic animals were carried out with one-factor or two-factor analysis of variance (ANOVA), based on the design of the experiment, with Bonferroni post-hoc tests. The nonparametric data resulting from assays of laser-ablated animals were analyzed by logistic regression.

Supplemental Data

Three figures are available at http://www.current-biology.com/cgi/content/full/17/21/1858/DC1/.

Acknowledgments

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Supplemental Data

Neural Sex Modifies the Function of a *C. elegans* Sensory Circuit

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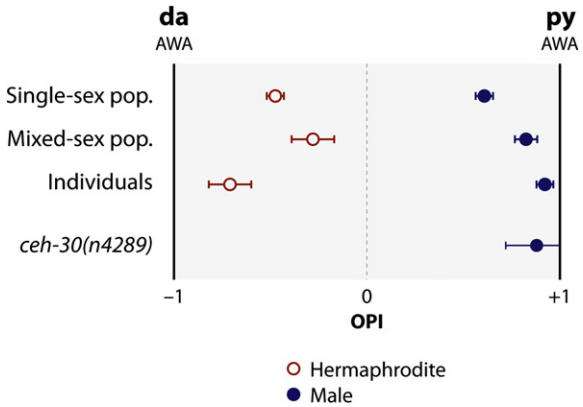

Figure S1. Sex Differences in Olfactory Preference Are Not By-Products of Male-Specific Behavior and Do Not Require the CEM Neurons

Data are shown for animals assayed in standard single-sex population assays, mixed-sex population assays, and animals assayed individually. *ceh-30* mutant males were assayed in standard, single-sex population assays. Each data point represents the weighted mean and weighted SEM of at least ten assays, each containing ~50 animals, except for *ceh-30*, which represents six assays of ~40 animals each.

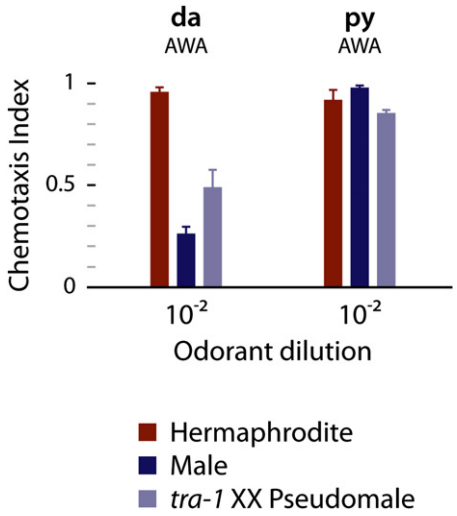

Figure S2. *tra-1* Pseudomales Display Male-like Attraction to da and py in the Single-Odorant Assay

Wild-type animals and *tra-1* XX pseudomales were tested for attraction to da and py at 1:100 with standard single-odorant assays. Each point represents the weighted mean and weighted SEM of at least four assays each containing ~50 animals. *tra-1* XX pseudomales displayed significant olfactory attraction to both odorants.

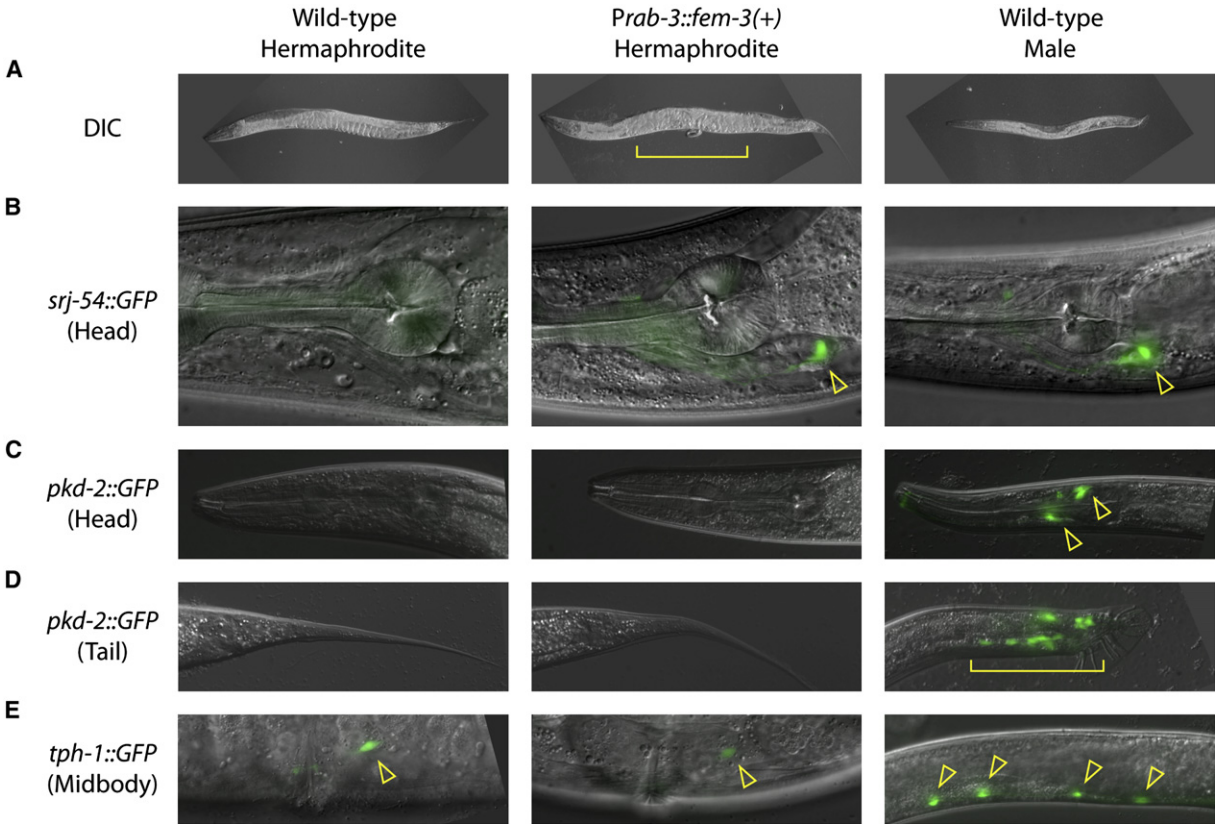

Figure S3. P*rab-3::fem-3(+)* Can Transform the Sex of the Core Nervous System but Has Minor Effects on the Sex-Specific Nervous System

(A) Whole-animal views of young adults showing a wild-type hermaphrodite, an *oxEx862* hermaphrodite, and a wild-type male. The transgenic hermaphrodite has a normal soma but retains extra eggs (bracketed area), indicating that the masculinization of the nervous system disrupts egg-laying behavior. A similar phenotype was seen in the P*osm-5::fem-3(+)* hermaphrodites (data not shown), indicating that this phenotype might stem from defects in hermaphrodite-specific sensory control of egg laying.

(B) Roughly half of *oxEx862* adult hermaphrodites express *srj-54::GFP* in the head AIM neuron (arrowhead). This expression is never seen in wild-type hermaphrodites but is observed in nearly all wild-type adult males.

(C) The *pkd-2::GFP* transgene marks the CEM neurons in the head of adult males (arrowheads). Expression is only very rarely observed in wild-type or *oxEx862* hermaphrodites, indicating that *oxEx862* does not result in the generation of CEM neurons in hermaphrodites.

(D) In the male tail, *pkd-2::GFP* is expressed in the male-specific RnB and HOB neurons (bracket). No expression was observed in the tails of wild-type or *oxEx862* hermaphrodites, indicating that these male-specific neurons do not form in *oxEx862* hermaphrodites.

(E) *tph-1::GFP* is expressed in the hermaphrodite-specific neuron HSN in wild-type and *oxEx862* hermaphrodites (arrowheads). Interestingly, *tph-1::GFP* expression is usually reduced in the HSNs of *oxEx862* hermaphrodites, and the HSN neurons in these animals are sometimes mispositioned (data not shown), indicating that *fem-3(+)* expression might disrupt HSN differentiation. We also sometimes observed *tph-1::GFP* expression in two cells flanking the vulva in *oxEx862* hermaphrodites; this might indicate that the hermaphrodite-specific cells VC4 and VC5 can adopt a CP-like fate in these animals. In adult males, *tph-1::GFP* marks these male-specific CP ventral cord neurons. Four of the CP neurons (open arrowheads) are visible in this view of a wild-type adult male.