Sexual Dimorphism and Sex Differences in *Caenorhabditis elegans* Neuronal Development and Behavior

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ABSTRACT As fundamental features of nearly all animal species, sexual dimorphisms and sex differences have particular relevance for the development and function of the nervous system. The unique advantages of the nematode *Caenorhabditis elegans* have allowed the neurobiology of sex to be studied at unprecedented scale, linking ultrastructure, molecular genetics, cell biology, development, neural circuit function, and behavior. Sex differences in the *C. elegans* nervous system encompass prominent anatomical dimorphisms as well as differences in physiology and connectivity. The influence of sex on behavior is just as diverse, with biological sex programming innate sex-specific behaviors and modifying many other aspects of neural circuit function. The study of these differences has provided important insights into mechanisms of neurogenesis, cell fate specification, and differentiation; synaptogenesis and connectivity; principles of circuit function, plasticity, and behavior; social communication; and many other areas of modern neurobiology.

KEYWORDS *Caenorhabditis elegans*; neurobiology; behavior; sex differences; sexual dimorphism; cilia; development; WormBook

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SINCE its development as a model system, *Caenorhabditis elegans* has been a rich source of insight into the biology of sex. The two sexes of the species, hermaphrodites and males, differ in many aspects, including development, anatomy, physiology, and behavior. In this review article, we focus on sex-specific and sexually modulated features of the nervous system and behavior. To complement and extend this material, we refer the reader to a number of other recent reviews on the biology of sex in and beyond the nervous system in *C. elegans* (Barrios 2014; Chute and Srinivasan 2014; Emmons 2014; Fagan and Portman 2014; García 2014; O'Hagan *et al.* 2014; Sherlekar and Lints 2014; García and Portman 2016; Portman 2017), as well as the recent description of the connectome of the *C. elegans* male tail (Jarrell *et al.* 2012). For a comprehensive description of sex-specific anatomy, see WormAtlas (www.wormatlas.org) and the companion *C. elegans* Atlas (Hall and Altun 2008).

Overview: Sex Differences in Neuroanatomy and Behavior

Like that of many other animals, the *C. elegans* nervous system is a central focus of the evolutionary forces that generate adaptive (and sometimes maladaptive) sex differences. Anatomically, sex differences in the *C. elegans* nervous system appear straightforward; superimposed onto a set of 294 shared, sex-common neurons are two sets of sex-specific neurons (Figure 1A). Eight such neurons in the hermaphrodite contribute to circuitry that enables egg-laying behavior, while 91 sex-specific neurons in the male subserve copulation and other male-specific behaviors. However, sexual dimorphism in the structure of the nervous system runs more deeply than this, as some shared neurons exhibit sex-specific structural and functional features; moreover, recent work has revealed sex-specific patterns of connectivity even among shared neurons.

The behavior of *C. elegans* also differs by sex in overt and subtle ways. Both sexes exhibit obvious sex-specific behaviors, most notably hermaphrodite egg laying and male mating. But many other aspects of behavior, including chemosensation, locomotion, and learning, are also modulated by biological sex. Furthermore, neuronal regulation of physiology and the production of chemical signals also differ between males and hermaphrodites. Together, these findings indicate that the roles of biological sex in circuit function and behavior go far beyond the programming of innate sex-specific behaviors.

Sex Determination and tra-1

The primary sex-determining cue in *C. elegans* is the ratio of X to autosomal chromosomes. In XX embryos, this ratio is high, specifying hermaphrodite development. In X0 animals, the X/A ratio is low, causing these individuals to develop as males. The genetics of sex determination in *C. elegans* is well-characterized and has been reviewed elsewhere (Zarkower 2006; Wolff and Zarkower 2008). The key relevant point is that this mechanism converges on a terminal "master regulator" of somatic sexual state, the transcription factor TRA-1A (Figure 1B). In the XX condition, levels of TRA-1A are high; in X0 animals, levels are low. Genetic evidence clearly shows that *tra-1* activity is necessary and sufficient to specify nearly all sex differences outside the germline: *tra-1*(*0*); *XX* animals are transformed into "pseudomales" in which all somatic tissues are fully masculinized, and *tra-1*(*gf*); *X0* animals are converted into females (Hodgkin 1987). As a transcription factor, TRA-1A acts cell-autonomously to regulate an array of targets that control more specific aspects of sex-specific development and physiology (Zarkower and Hodgkin 1992). While some of these are known, many remain unidentified (Berkseth *et al.* 2013). Interestingly, several factors that act directly (or indirectly) downstream of TRA-1A are members of the DM (*doublesex* and *mab-3*) family, orthologs of *doublesex* (*dsx*), a master regulator of sexual differentiation in *Drosophila* (Matson and Zarkower 2012) [interestingly, the control of sex differences in the fly nervous system has largely been outsourced to an insect-specific factor, *fruitless*,

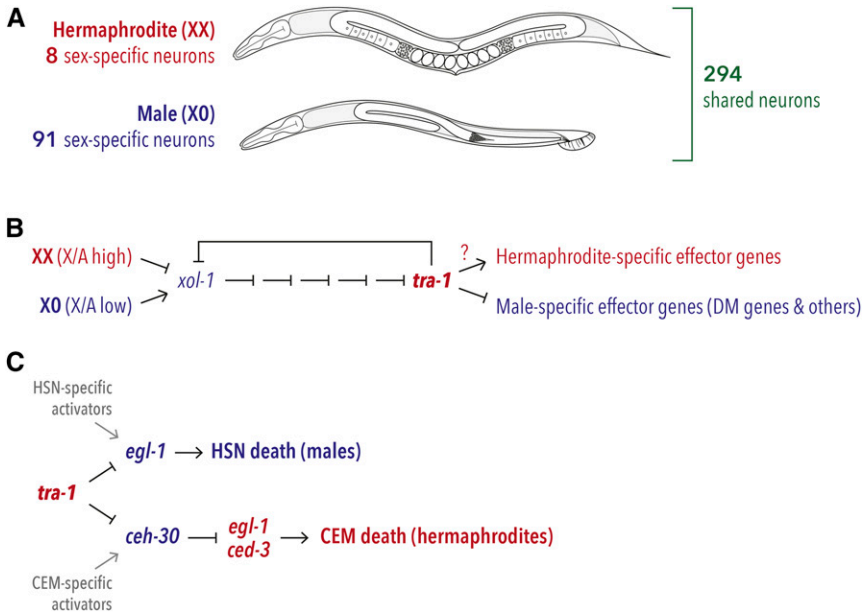

Figure 1 Sex differences in the *C. elegans* nervous system. (A) The adult XX hermaphrodite has eight sex-specific neurons (the two HSNs and six VCs), while the adult male has 91 sex-specific sensory, inter, and motor neurons. These sex-specific neurons are densely interconnected with 294 sex-shared neurons; cells that are lineally and anatomically analogous between the two sexes. (B) The primary sex-determining cue in *C. elegans*, the X-to-autosome ratio (X/A), sets the state of the genetic sex-determination pathway, a simplified version of which is shown here. Ultimately, the X/A ratio determines whether *tra-1*, the master regulator of essentially all somatic sexual characteristics in *C. elegans*, is active or inactive. Active *tra-1* represses numerous male-specific genes, including several members of the *doublesex*-like DM domain family. *tra-1* may also directly promote hermaphrodite-specific characteristics, but this is less-well-understood. (C) Sex-specific programmed cell death generates sexual dimorphism in the survival of the hermaphrodite-specific HSN neurons and the male-specific CEM neurons. In the HSNs, *tra-1* acts in hermaphrodites to repress the cell death activator *egl-1*, allowing them to survive in hermaphrodites. In the CEMs, *tra-1* represses *ceh-30*, itself an inhibitor of the cell death genes *egl-1* and *ceh-30*. This provides for male-specific survival of the CEMs.

though *dsx* does have important roles in regulating neurogenesis (Robinett *et al.* 2010)]. The identification of TRA-1A targets, and the connection of these to the sex differences in neural development and function discussed below, remains an area of active interest.

Development and Anatomy: Sexual Dimorphism in Cell Lineage and Differentiation

As described above, sex differences in the *C. elegans* nervous system occur at multiple levels. Some aspects of sexually dimorphic neuronal and neuromuscular anatomy arise through simple changes in patterns of cell lineage (precursor proliferation, fate specification, and programmed cell death). Other kinds of sexually dimorphic features, such as neuronal connectivity and ciliary specialization, can be seen as cases of sexual regulation of differentiation and physiology.

Sex-specific cell death: CEMs and HSNs

Perhaps the most developmentally straightforward origin of sexually dimorphic neuroanatomy can be seen in the cases of the hermaphrodite-specific HSN and male-specific CEM neurons (Sulston *et al.* 1983). The HSNs are a pair of serotonergic motor neurons in the midbody; they play a key role in triggering egg-laying events (see below). The four CEMs detect hermaphrodite-derived pheromone and, along with the non-sex-specific CEP neurons, innervate the cephalic sensilla (see below). For both HSNs and CEMs, sexual dimorphism arises through sex-specific programmed cell death: these neurons are born in embryos of both sexes, but apoptosis is triggered only in males (HSNs) or hermaphrodites (CEMs) (Figure 1C). In the HSNs, the proapoptotic gene *egl-1* is repressed in XX animals through direct regulation by TRA-1A. In X0 animals, the lack of *tra-1* function allows *egl-1* to be expressed in the embryonic HSN, causing their death (Conradt and Horvitz 1999). For the CEMs, a conceptually similar but slightly embellished mechanism operates. Here, TRA-1A represses the transcription factor CEH-30 in hermaphrodites; CEH-30 activity in males then promotes their survival by repressing the expression of *egl-1* and the caspase *ced-3* (Peden *et al.* 2007; Schwartz and Horvitz 2007; Nehme *et al.* 2010). Interestingly, the HSNs and CEMs are the only sex-specific neurons that are born embryonically; all others arise through sex-specific alterations in larval lineages. Infrared laser induction of *hsp-16.2*::myr_mCherry expression in CEMs of embryonic hermaphrodites and subsequent time-lapse imaging reveals that these neurons extend a dendrite before dying (Singhal and Shaham 2017). However, complete differentiation of the HSNs and CEMs is arrested until late larval development (Sulston and Horvitz 1977; Sulston *et al.* 1980), perhaps because their functions are necessary only in adults. The mechanisms regulating this arrest and reactivation are not well-described, though the heterochronic pathway has been implicated in controlling the timing of HSN differentiation (Olsson-Carter and Slack 2010).

Sex-specific neurogenesis: regulation of cell lineage and differentiation

Several other classes of sex-specific neurons are generated through relatively simple changes in cell lineage during larval development. In the ventral nerve cord, sex-specific neurons are present in both hermaphrodites and males. In hermaphrodites, these are the six VC neurons, cholinergic

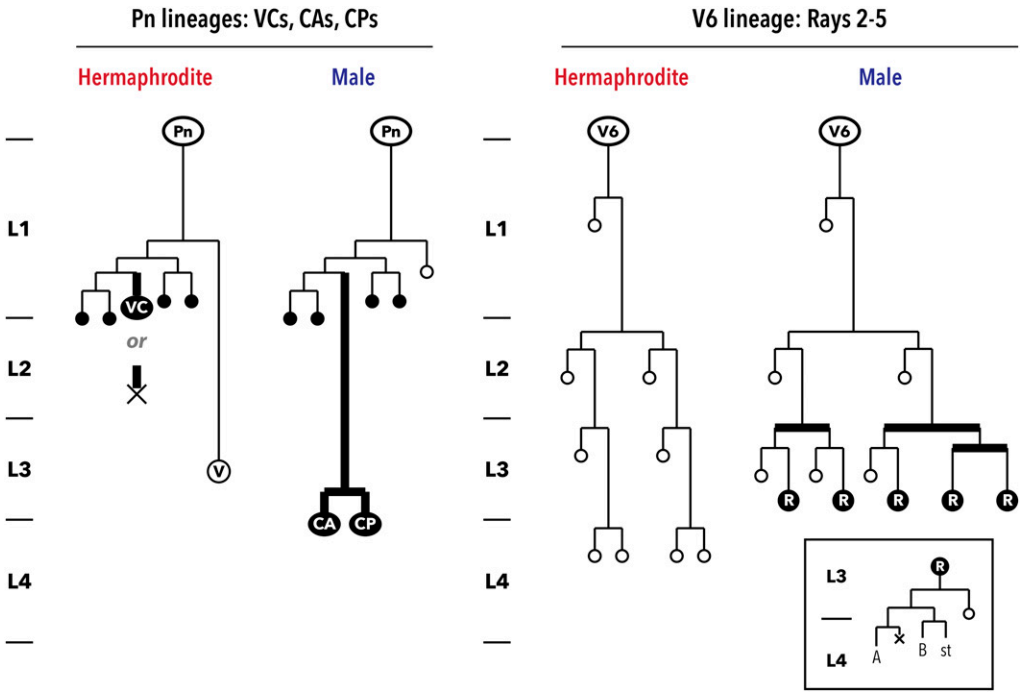

Figure 2 Development of sex-specific neurons: lineage alterations. Left: the Pn.a lineages generate several classes of shared neurons (filled circles) as well as three classes of sex-specific neurons, the VCs in hermaphrodites [or programmed cell deaths (X) in the most anterior and posterior Pn lineages] and the CAs and CPs in males. Sexual dimorphism in the Pn.aap lineage, shown in bold lines, involves the male-specific division of Pn.aap and the sexually dimorphic establishment of cell fates. The Pn.p cells also exhibit sexual dimorphism; in the midbody, these cells contribute to vulval lineages (V) in the hermaphrodite but retain hypodermal fates (open circle) in the male. Right: ray development involves sexual dimorphism in the lineages of three posterior seam cells, V5, V6, and T. Only V6, which generates five of the nine pairs of rays, is shown here. Two key male-specific features of the V6 lineage are the doubling divisions in L3 (bold horizontal lines) and the specification of the ray precursor cells (R). The ray sublineage, shown in the boxed inset, is a program of division and differentiation executed by each ray precursor cell to generate the three cell types of each ray, as well as a cell that dies (X) and a hypodermal cell.

motor neurons that work together with the HSNs to regulate egg laying (see below). In contrast, males possess two classes of sex-specific ventral cord neurons, the CAs and CPs. The function of neither of these classes is well-understood, but they are both implicated in male copulatory behavior (see below). Like other ventral cord motor neurons, the VCs, CAs, and CPs arise among the progeny of the ventral hypodermal Pn.a cells. These cells, arrayed along the length of the worm's ventral surface, execute nearly identical, stereotyped lineages to give rise to all of the major classes of ventral cord motor neurons (Sulston and Horvitz 1977). Only one of the cells in this lineage, Pn.aap, exhibits overt sexual dimorphism (Figure 2). In hermaphrodites, this cell differentiates into a VC neuron or, in the anterior- and posterior-most regions of the body, undergoes programmed cell death. However, in males, nearly all of the Pn.aap cells divide once, giving rise to one CA and one CP neuron (again, lineages at the extreme ends of the ventral hypodermis are slightly modified). The regulatory mechanisms that implement this sexual dimorphism in lineage and cell fate remain largely unclear. Several factors contribute to patterning and fate specification in both sexes, including the Hox genes *lin-39* and *mab-5*, and the TALE-homeodomain transcription factor genes *unc-62* and *ceh-20* (Clark *et al.* 1993; Salser *et al.* 1993; Liu *et al.* 2006; Potts *et al.* 2009; Kalis *et al.* 2014). It seems likely that TRA-1A acts together with these and other factors in the ventral hypodermis to control its sexually dimorphic development.

A second case of sex-specific neurogenesis in the context of a shared developmental lineage can be found in the rays, 18 bilateral sensilla that decorate the cuticular fan of the male tail (Figure 2). Like the ventral cord motor neurons, the rays are also generated from an array of hypodermal cells along the anterior–posterior axis; however, in this case it is the lateral hypodermal cells (the seam cells), rather than ventral cells, that act as neural progenitors (Sulston *et al.* 1980). In both sexes, most seam cells undergo a repeated stem cell-like division program during larval development, dividing asymmetrically to regenerate themselves and to also produce a cell that joins the growing hyp7 hypodermal syncytium. However, the lineages of the posterior seam cells V5, V6, and T undergo male-specific alterations to allow ray development. First, an extra doubling division takes place in the V6 lineage during L2; this requires the Hox gene *mab-5*, but it is not known how *tra-1* specifies this event (Salser and Kenyon 1996). Second, during L3, the *atonal*-class bHLH (basic helix-loop helix) transcription factor LIN-32 is activated male-specifically in nine pairs of ray precursor cells (Rn cells) generated by the V5, V6, and T lineages (Zhao and Emmons 1995). *lin-32* expression requires the Hox genes *mab-5* and *egl-5* (Zhao and Emmons 1995), as well as the DM gene *mab-3*, which indirectly activates *lin-32* by repressing its repressor *ref-1* (Shen and Hodgkin 1988; Yi *et al.* 2000; Ross *et al.* 2005). However, here again, how *tra-1* restricts *lin-32* expression to males is not fully understood. Once expressed, *lin-32* and its dimerization partner *hlh-2* are necessary and likely sufficient to trigger the ray sublineage (Zhao and Emmons 1995; Portman and Emmons 2000). This series of asymmetric cell divisions is executed by each

of the Rn cells, giving rise to the three cells of each ray [two neurons (A-type and B-type, or RnA and RnB) and a glial structural cell (Rnst)], as well as a hypodermal cell (Rn.p) and an apoptotic cell (Rn.aap). A Wnt/β-catenin signaling pathway acts to generate asymmetric cell fate decisions within the ray sublineage, and *lin-32* and *hlh-2* also play later roles in the differentiation of the ray neurons themselves (Portman and Emmons 2000, 2004; Miller and Portman 2011). Further patterning mechanisms, including the TGFbeta-family ligand DBL-1, generate unique identities among the rays (Chow and Emmons 1994; Lints and Emmons 1999; Lints *et al.* 2004; Choy *et al.* 2007; Wong *et al.* 2010). While the exact sensory modalities of the ray neurons remain unknown, behavioral studies indicate that they participate in multiple steps of male mating behavior (see below), and likely have both mechanosensory and chemosensory functions.

The ventral hypodermis and the seam are not the only sites of sex-specific neurogenesis, as several precursor cells in the tail (B, F, Y, and U) give rise to male-specific neurons; these lineages are described below. A further (and surprising) instance of sex-specific neurogenesis is that of the MCMs (for "mystery cells of the male"). Though the *C. elegans* community had for years been confident that the complete cellular anatomy of the nervous system was known, a recent study disrupted this sense of security by discovering an unnoticed pair of interneurons in the head of the *C. elegans* male (Sammut *et al.* 2015). These cells, the MCMs, arise male-specifically during larval development. This happens through an unprecedented mechanism: the MCMs are the daughters of a pair of fully differentiated glial cells, the amphid sheath socket (AMso) cells, which undergo male-specific mitosis late in larval development to produce the two MCMs while maintaining the two AMso glia. The genetic sex of AMso determines whether this division will occur, as genetic masculinization of this cell is sufficient to generate MCMs in hermaphrodites. Interestingly, MCM neuron function is necessary for "sexual conditioning," a process in which males learn to use sodium chloride as a cue to help locate mates (see below) (Sakai *et al.* 2013; Sammut *et al.* 2015).

As this review was being written, the discovery of yet another "new" class of male-specific neurons, the PHD cells, was emerging (B. Kim, S. Emmons, R. Poole, and A. Barrios, personal communication). We expect studies of the development and function of these neurons to reveal additional fresh insights into *C. elegans* neurobiology.

Sexual dimorphism in the hindgut: sex-specific development and organogenesis

A prominent dimorphism between the sexes is the post-embryonic development of somatic cells that give rise to the hindgut, an epithelial channel that connects the posterior intestine to the outside of the worm, and also produce a substantial cohort of male-specific neurons (see WormAtlas, www.wormatlas.org/male/alimproct/Procframeset.html). In hatched L1 hermaphrodite and male larvae, the hindgut is composed of six epithelial rectal cells: K, K′, B, F, Y, and U (Sulston *et al.* 1983). However, early in L1 larval development, dimorphism occurs in some of these cells (Sulston and Horvitz 1977; Sulston *et al.* 1980). The hermaphrodite Y cell changes its rectal cell fate to the PDA neuron during L1 development, whereas B, U, and F remain as single rectal epithelial cells throughout the hermaphrodite's life. In contrast, the male B, F, Y, and U cells further divide to eventually form reproductive-related structures and neurons (Figure 3A).

Some of the male hindgut blast cells divide during L1, but the majority of cell divisions occur throughout L2 and L3 stages. The first male hindgut cells to divide are Y and B, which asymmetrically divide anteriorly and posteriorly during L1 (Figure 3, A and B). The two Y cell daughters become the male PDA neuron and the blast cell Y.p. During the L2 and L3 stages, Y.p undergoes further divisions to generate the neural and structural cells of the postcloacal sensilla. The male F and U blast cells also begin their divisions at the L2 stage (Figure 3B). At the end of L3, the F descendants generate the sex-specific dorsal rectal ganglion neurons EF1, EF2, DX1, DX2, and two cells that form part of the adult male proctodeum. The proctodeum is the adult male's modified rectum that includes the posterior alimentary conduit, the cloaca, the gonadal vas deferens–cloacal junction, the copulatory spicules, and their associated sex-specific neurons. The U cell descendants generate the sex-specific preanal ganglion neurons EF3, EF4, DX3, and DX4, as well as cells (U.Lp and U.Rp) that aid in connecting the male somatic gonad to the proctodeal channel. Also during the L2 and L3 stages, the male B cell lineage generates a large number of descendants (47 cells) that form the proctodeum and its associated copulatory structures.

During L1, the first asymmetric division of the B cell specifies the cell lineages that will form the wall of the proctodeal/cloacal cavity, and the structural and neural components of the copulatory spicules (Figure 3C). Lineages derived from the B.a anterior daughter give rise to 37 cells of multiple types: proctodeal cells, spicule socket cells, neural sheath cells, and spicule sensory neurons. In contrast, the posterior B.p daughter cell produces fewer progeny (five cells); these function as proctodeal cells, interneurons, or part of the hypodermis. The initial asymmetric B.a and B.p lineages are specified by the combined actions of the *vab-3* (a Pax gene family homolog) and *egl-5* (an *Abdominal B* Hox gene homolog) transcription factor genes (Johnson and Chamberlin 2008), in conjunction with the WNT planar cell polarity signaling pathway (Wu and Herman 2006; Wu *et al.* 2007). The B.p identity is the default fate for both daughters of B; however, the *vab-3* gene product can promote adoption of B.a characteristics in both. The *vab-3* domain of expression, which includes both B.a and B.p, is initially specified by the posterior patterning Hox transcription factor EGL-5. The restricted expression of *vab-3* to B.a results from LIN-44/WNT ligand and LIN-17/frizzled receptor planar cell polarity signaling that inhibits *vab-3* expression in B.p. Consistent with this, mutations in *lin-17* cause both B daughter cells to express *vab-3* and adopt B.a characteristics

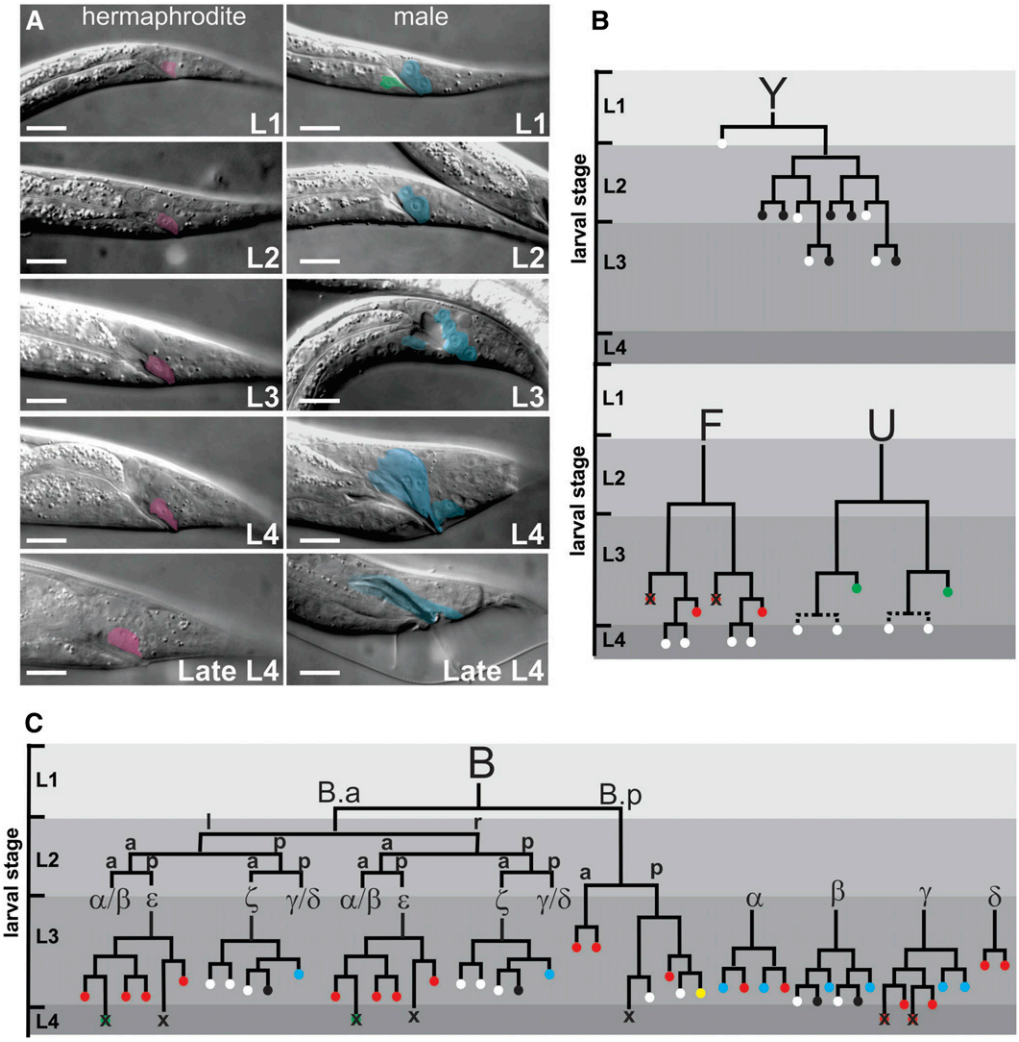

Figure 3 Development of cellular structures associated with the male hindgut. (A) Left and right panels display DIC images of the rectal region in the hermaphrodite and male, respectively, at different developmental stages. The B cell is shaded pink in the hermaphrodite. The B cell descendants are shaded blue in the male. In the L1 male, the blast cell Y.p is shaded green. The scale bar represents 10 μm; dorsal is to the top and anterior is to the left. (B) Division patterns of the male Y, F, U, and (C) B blast cells. Abbreviations for the L1 and L2 B lineages: anterior, a; posterior, p; left, l; and right, r. The black circles represent structural cells associated with neurons; white circles depict neurons; blue circles represent spicule socket cells; the yellow circle represents a cell that fuses with the hypodermis; red circles depict proctodeal cells; circles with an X depict cells that variably die; X represents cells that are programmed to die; dashed lines depict cells that variably divide; and green circles represent cells that participate in connecting the gonadal vas deferens to the proctodeum. For the B lineage, the α, β, γ, and δ lineages were expanded separately from the rest of the lineage, since the fates of the L2 cells B.a(l/r)pp and B.a(l/r)aa are not invariant.

(Wu and Herman 2006). *lin-44* is expressed in the posterior hypodermal tissue, which is spatially closer to the posterior B daughter cell. The proximity of the *lin-44*-expressed WNT ligand to B.p likely accounts for the asymmetric *vab-3* expression in B.a (Johnson and Chamberlin 2008).

During L2 stage, the B.a lineage undergoes three additional divisions. In this lineage, cell fates are influenced by concerted interactions among B.a descendants, and with the neighboring F, U, and Y.p cells and/or their descendants (Chamberlin and Sternberg 1993). These interactions involve inductive and inhibitory positional cues, modulating signals, and reciprocal lateral signaling among potentially equivalent cells. The F and U cells promote anterior identities, whereas Y.p and interactions between the B.a progeny promote posterior identities. Here, *lin-3* (EGF)/*let-23* (EGF receptor)/*let-60*(RAS), *lin-12* (Notch), and *dbl-1* (TGFβ) signal transduction pathways specify the differential identities of the eight B.a descendants, α, β, γ, δ, ε(left/right), and ζ(left/right) (Chamberlin and Sternberg 1994). For one of these descendants, B.aγ, the cell-specific expression of *ceh-13* (*labial* Hox gene homolog) is dependent on both *lin-3* and *dbl-1* signaling (Stoyanov *et al.* 2003; Seah and Sternberg 2009).

The lineages of the eight B.a descendants go through their final two or three divisions during the L3 stage. Similar to the L1 and L2 stages, EGF, TGFβ, Notch, and WNT signal transduction pathways promote the terminal cell fates of the B progeny (Seah and Sternberg 2009). During L4, these cells undergo their developmental and functional maturation, causing the male tail to radically change its form. In conjunction with B.p descendants, eight B.a progeny will form the proctodeal/cloacal cavity. The remainder of the B.a descendants form the copulatory spicules, the organs used to penetrate the hermaphrodite vulva during copulation. Four B.a progeny will become sensory-motor neurons, SPC(L/R) and PCC(L/R), which stimulate the contractions of sex-specific muscles used for copulation. Four others will become the bilateral spicule sensory neurons SPV(L/R) and SPD(L/R); a further four will become the SPsh spicule sheath cells; and eight (two groups of four) will become the syncytial SPso spicule socket cells, which encase the sheath cells and the sensory processes of the SPV and SPD neurons (Sulston *et al.* 1980).

During mid-L4-to-late stage, the spicule socket cells (derived from members of the α, β, γ, and δ lineages) form the

elongated sclerotized blade-like structure of the spicules. Physical interactions between the socket cells and the developing male sex muscles are required for proper spicule development. The socket cells, as well as other cells of the proctodeum, secrete the *egl-17*-encoded FGF-like ligand, which promotes the developing sex muscles to position themselves properly with the proctodeal region; however, *egl-17* mutants develop normal spicules, indicating that additional secreted molecule(s) must act in conjunction with FGF (Jiang and Sternberg 1999). Spicule formation also requires the socket cells and proctodeum to elongate anterodorsally. This process requires the TGFβ components *daf-4*, *sma-2*, *sma-3*, and *sma-4*, and the *rnt-1/mab-2*, *lin-31*, and *lin-29* transcription factors (Baird and Ellazar 1999; Euling *et al.* 1999; Suzuki *et al.* 1999; Jia *et al.* 2004; Kagoshima *et al.* 2005). Mutations in any of these signaling components or laser ablation of the male sex myoblast cells will disrupt anterodorsal elongation and cause the spicules to develop into stunted, crumpled structures. After anterodorsal elongation, the socket cells secrete a material between themselves and the adjacent proctodeal cells that will harden to form the sclerotized cuticle shell of the spicules (Jiang and Sternberg 1999). Finally, in L4 lethargus, both sets of four socket cells fuse to form two syncytia.

The male vas deferens and the hook sensillum, structures associated with the B cell-derived proctodeum, are unique in the male; however, they share some developmental similarities with hermaphrodite-specific tissues. The vas deferens is the conduit that allows sperm to travel from the gonadal seminal vesicle to the proctodeal/cloacal opening. The male linker cell guides the male germline and somatic gonad to the ventral posterior region during L2–L4 stages of development (Figure 4A). At mid- to late-L4 stage, the linker cell reaches the proctodeal/cloacal region (Figure 4B) and undergoes a nonapoptotic cell death program in which it is later engulfed by one of the U-derived progeny (either U.Lp or U.Rp) (Figure 4C). The death of the male linker cell does not involve the *ced-3*-encoded caspase or other canonical apoptosis pathway genes, but instead uses the heat shock factor HSF-1 and the ubiquitin proteasome system. EGL-20/LIN-44 WNT ligands, LIN-29-mediated transcriptional regulation, and the TIR-1/SEK-1 kinase pathway provide three redundant systems that regulate the positional timing of the male linker cell's death (Abraham *et al.* 2007) (Blum *et al.* 2012; Kinet *et al.* 2016). After the linker cell dies and its corpse is engulfed, one of the proctodeal cells, B.aε(left or right)aav, directly connects the vas deferens' opening to the proctodeal cavity. The B.aε(left or right)aav cell requires the *cog-1*-expressed GTX/Nkx6.1-like homeobox transcription factor to mediate the connection of the two tissues (Palmer *et al.* 2002). Interestingly, the hermaphrodite also uses this transcription factor to connect the developing uterus with the vulval channel. In the developing L4 hermaphrodite vulva, the vulC, vulD, vulE, and vulF cells express *cog-1*. During development, the anchor cell of the hermaphrodite uterus positions itself over the vulF cells, which then separate to form an opening. The anchor cell

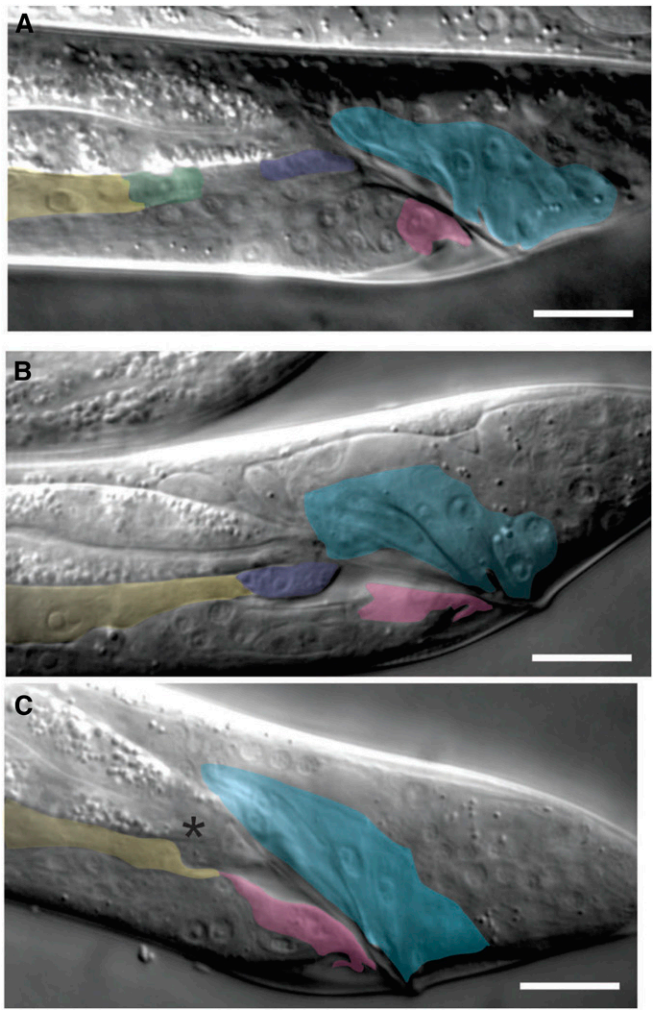

Figure 4 Connection of the vas deferens to the proctodeal/cloacal region. (A) DIC image of an early mid-L4 male. The migrating vas deferens, shaded in yellow, is ~15–20 μm from the proctodeal/cloacal channel. The linker cell is shaded in green; one of the U blast cell progeny, either U.lp or U.rp, is shaded in purple. The hook cell is shaded in pink and the B-derived cells that form the posterior proctodeum are shaded in blue. (B) DIC image of the vas deferens (yellow) reaching either U.lp or U.rp (purple). The dying linker cell is not in the focal plane. (C) The vas deferens is connected to the cloacal channel; the linker cell corpse can partially be seen (asterisk). Bar, 10 μm; dorsal is to the top and anterior is to the left.

fuses with the neighboring uterine cells (the utse cells), so that a thin membrane overlays the vulF opening. The membrane is ruptured in the adult when the first egg is forced out from the uterus cavity and through the vulva (Newman and Sternberg 1996). In *cog-1* mutant hermaphrodites, the vulF cells do not separate, thus blocking the opening between the uterus and the rest of the vulval channel (Palmer *et al.* 2002).

The development of the male hook sensillum also shares similarities with the development of the hermaphrodite vulva. The male hook sensillum is located beneath the vas deferens and immediately anterior of the proctodeal/cloacal opening. This tissue is made up of a sclerotic barbed appendage called the hook, the sensory endings of the HOA and HOB sensory neurons, a neural sheath cell, and a socket cell. The hook

sensillum functions to detect chemical and physical features of the hermaphrodite vulva, and possibly to also scrape away copulatory plugs that may obstruct the vulval opening (Liu and Sternberg 1995; Garcia *et al.* 2007). The hook sensillum develops from an equivalence group comprised of the blast cells P9.p, P10.p, and P11.p. In the hermaphrodite, these cells fuse with the hyp7 syncytium. Reminiscent of the hermaphrodite vulval developmental program, blast cell progeny develop in primary, secondary, and tertiary patterns: the progeny of P11.p adopt the primary fate (preanal ganglion neurons), P10.p progeny adopt the secondary fate (the hook sensillum), and P9.p is the tertiary fate (hypodermal). In vulval development, LIN-3/EGF signaling specifies primary and secondary fates (the vulval channel and lips), and LIN-12/NOTCH signaling patterns secondary fates. In contrast, the male uses Wnt signaling via LIN-17/Frizzled receptors to specify and execute both primary and secondary fates; LIN-12/NOTCH-mediated lateral signaling is used to fine-tune the pattern and induce the hook sensillum secondary fate (Yu *et al.* 2009, 2010).

Sexual dimorphism in reproductive musculature

The sex-specific muscles, associated with the hermaphrodite vulva, and the male tail and spicules, are additional examples of dimorphic development. In both sexes, these muscles are derived from the shared embryonic-derived M blast cell. During early and mid-L1 in both sexes, the M cell descendants follow similar division patterns. At the end of the L1 stage, the hermaphrodite M cell produces 14 additional body wall muscle cells, two coelomocyte cells, and two sex myoblast cells (Figure 5A). During the L2 stage, the bilateral pair of sex myoblast cells uses the products of gonadal-expressed *egl-17*/FGF (Burdine *et al.* 1998; Branda and Stern 2000) and the myoblast-expressed *egl-15*/FGF receptor (Lo *et al.* 2008) to migrate anteriorly to the somatic gonadal region, and undergo further divisions in L3 to form the eight uterine and eight vulval muscle cells. The uterine muscles function to squeeze eggs from the uterus and the vulval muscles function to widen the vulval channel (Figure 5, B and C). The larval male's M cell divisions parallel the hermaphrodite's program, forming 14 body wall muscle cells and two sex myoblasts; however, at the end of the male L1 stage, the two descendants, which in the hermaphrodite terminally develop into coelomocytes, instead divide once more to form additional sex myoblasts. Hence, the male develops six myoblasts, in contrast to the hermaphrodite's two. The male sex myoblasts migrate posteriorly to the tail and further divide in the L3 stage to form 41 genital muscles and one coelomocyte (Figure 6A). In contrast to hermaphrodites, mutations in *egl-17*/FGF-like ligand do not affect male sex muscle morphology, but mutations in the *egl-15*/FGF receptor do cause variable sex muscle abnormalities (Trent *et al.* 1983), suggesting that the male uses these signaling molecules differently than the hermaphrodite.

During the L4 stage, the male muscles form their contractile specializations to control tail postures, spicule movements,

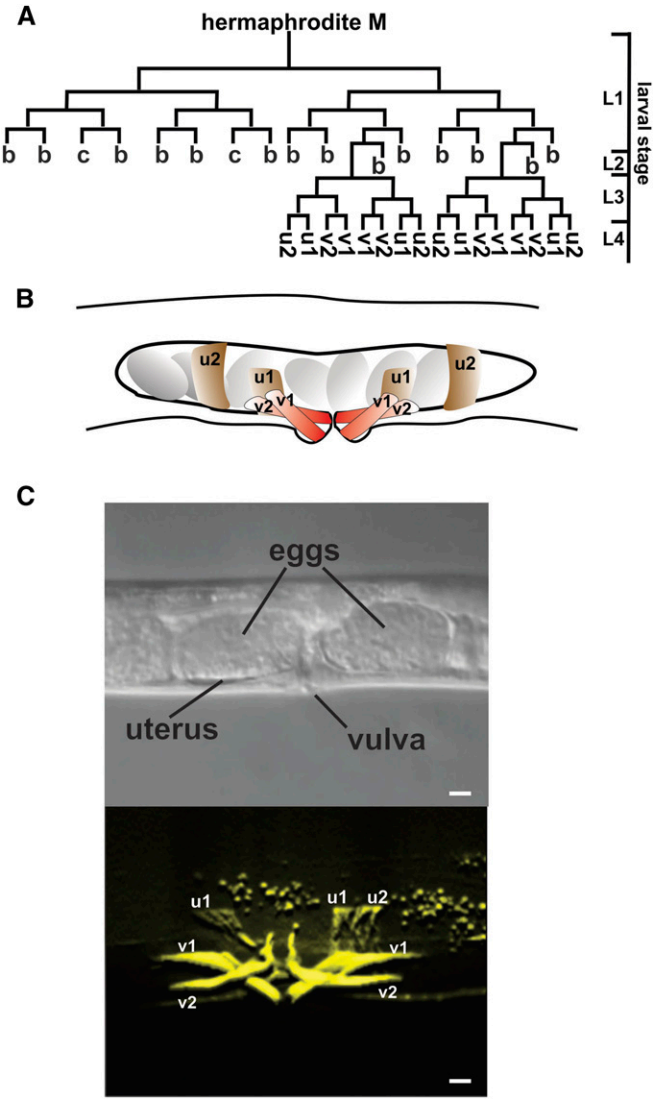

Figure 5 Hermaphrodite reproductive musculature. (A) Developmental lineage of the hermaphrodite M cell. The right bar shows larval stages. Abbreviations: b, body wall muscle; c, coelomocyte; u1, uterine muscle 1; u2, uterine muscle 2; v1, vulva muscle 1; and v2, vulva muscle. (B) Lateral cartoon depiction of the hermaphrodite vulva and uterine muscles. (C) Top panel: DIC image of the vulval/uterine region of a young hermaphrodite adult. Bottom panel: left lateral to medial flattened Z-stack confocal fluorescence image of the hermaphrodite vulva and uterine muscles. The muscle contractile fibers are labeled with YFP:actin expressed from the *unc-103E* promoter. Bar, 10 μm; dorsal is to the top and anterior is to the left.

and widening of the proctodeal/cloacal channel (Figure 6, B and C). Ten male-specific muscles (the caudal, outer, and inner longitudinal muscles) are associated with the sex-shared body wall muscles. Located at the ventral region of the tail, a bilateral set of 15 diagonal muscles (eight on the right and seven on the left, located to the fore of the cloaca) and four oblique muscles (two on both sides, located to the rear of the cloaca) facilitates the range of ventral body flexures the male performs during copulation. Connected to each spicule are two protractor (dorsal and ventral) and two

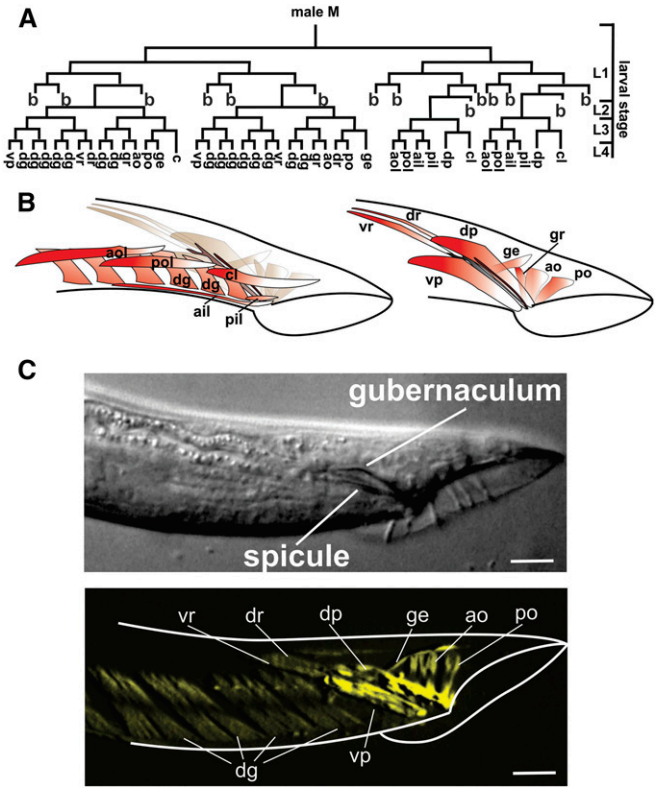

Figure 6 Male reproductive musculature. (A) Developmental lineage of the male M cell. The right bar shows larval stages. Abbreviations: b, body wall muscle; c, coelomocyte; vr, ventral retractor; dr, dorsal retractor; ao, anterior oblique; po posterior oblique; ge, gubernaculum erector; gr, gubernaculum retractor; aol, anterior outer longitudinal; pol, posterior outer longitudinal; ail, anterior inner longitudinal; pil, posterior inner longitudinal; and cl, caudal longitudinal. (B) Lateral cartoon depiction of the male copulatory muscles. (C) Top panel: left lateral DIC image of the young male adult. Bottom panel: left lateral to medial flattened Z-stack confocal fluorescence image of the male sex muscles. The muscle contractile fibers are labeled with yellow fluorescent protein::actin expressed from the *unc-103E* promoter. Bar, 10 μm; dorsal is to the top and anterior is to the left.

retractor (dorsal and ventral) muscles that control the eversion of the spicules from the cloacal opening. Also associated with the cloacal opening are the anterior and posterior inner longitudinal muscles. The functions of these muscles are not yet known, but they are situated to facilitate widening of the cloacal orifice as the male attempts to extend spicules into the hermaphrodite. Lining the posterior proctodeal cavity is a thin V-shaped cuticular strip called the gubernaculum. In a process similar to the formation of the spicules, the gubernaculum cuticle is secreted by the proctodeal cells B.paa (Jiang and Sternberg 1999). Four gubernaculum muscles (two on each side) are attached to the gubernaculum. During copulation, the gubernacular muscles pull the gubernaculum and the associated proctodeum posteriorly, widening the proctodeal/cloacal cavity, which facilitates the protraction of the spicules and subsequent sperm transfer (Liu *et al.* 2011; LeBoeuf *et al.* 2014).

An additional conspicuous dimorphic alteration that occurs in the L4 larval male is the remodeling of the sex-shared sphincter muscle and the anal depressor muscle. In hermaphrodites and L1–L3 larval males, the sphincter muscle is situated at the junction between the intestine and the hindgut, whereas the anal depressor is an H-shaped cell that contains left and right sarcomeres that are attached to the rectal epithelia [the F and B cells of the hermaphrodite hindgut and B.p (and later B.pa) of the larval male] and the dorsal body wall (Figure 7, A–C). In these animals, both the sphincter and anal depressor are enteric muscles that facilitate the expulsion of fecal waste. In contrast, the role of the adult male's sphincter muscle in defecation behavior is slightly different from that of the hermaphrodite and L1–L3 male. Likewise, the adult anal depressor muscle does not even function in defecation, but instead facilitates spicule extension and sperm transfer during copulation (Figure 7A') (Sulston *et al.* 1980; Reiner and Thomas 1995; LeBeouf and Garcia 2017).

The sequence involved in defecation behavior is mostly similar between hermaphrodites and males. In both sexes, defecation behavior occurs in three steps: posterior body wall contraction (pBoc), anterior body wall contraction (aBoc), and expulsion (exp). Intestinal Ca^{2+} oscillations and neuronal dense-core vesicle release regulate the pBoc and aBoc steps, respectively (Peters *et al.* 2007; Speese *et al.* 2007; Beg *et al.* 2008). The pBoc and aBoc behavioral steps move gut contents to the posterior intestinal region. The intestine also regulates the exp step. The intestine secretes *nlp-40*-encoded peptides, which in turn stimulate *aex-2*-encoded G-protein-coupled receptors (GPCRs) on the γ-aminobutyric acid (GABA)ergic defecation motor neuron DVB (Thomas 1990; Mahoney *et al.* 2008; Wang *et al.* 2013). GABA secretion subsequently stimulates *exp-1*-encoded excitatory channels on the intestinal, sphincter, and anal depressor muscles to induce muscle contraction (Beg and Jorgensen 2003). In hermaphrodite and L1–L3 males, the contracted intestinal and sphincter muscles isolate the fecal waste to the hindgut region, that is, the region between the sphincter muscle at the posterior end of the intestine and the anus. Since the anus of the hermaphrodite and of the L1–L3 male is closed to the outside, the opening of the anus is coordinated with the previous steps of defecation behavior. The anal depressor's bilateral sarcomeres are oriented in the dorsal and ventral axis, which, when stimulated to contract via GABA signaling, lifts the rectal epithelia so that the fecal waste will subsequently pass through the anal opening.

In contrast to the hermaphrodite and L1–L3 larval males, the adult male proctodeal anal cavity and cloacal orifice develop from the larval rectal epithelial cells. As a consequence, the cavity of the male's hindgut is opened to the environment. Prior to defecation, the intestinal opening must be sealed to keep gut contents from leaking out. Thus, during L4 development, the sphincter muscle undergoes muscle hypertrophy, pinching closed the intestine from the hindgut (Sulston *et al.* 1980). Because of these changes, the adult male alters the execution of the defecation exp step. The adult hypertrophic sphincter muscle reverses its response to GABA, such that this neurotransmitter induces muscle relaxation, rather than

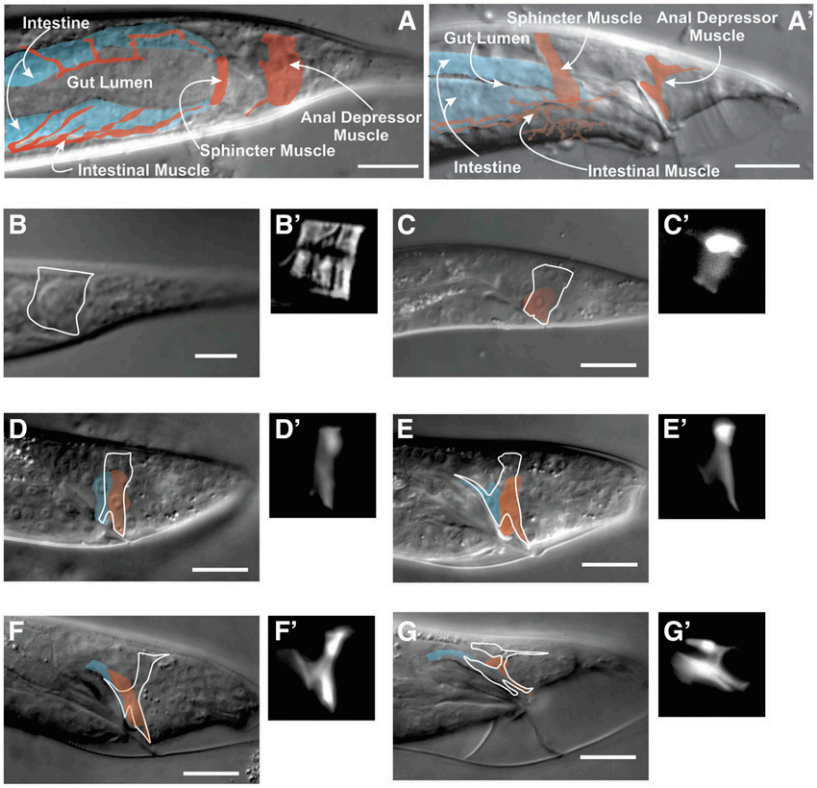

Figure 7 Hermaphrodite and male anal depressor. DIC lateral images of (A) adult hermaphrodite and (A′) adult male. The colored fill-ins depict the approximate shape and location of the animal's intestine, gut lumen, intestinal muscle, sphincter muscle, and anal depressor muscle. (B) DIC image of an adult hermaphrodite tail. (B′) Left lateral to medial flattened Z-stack confocal fluorescence images of the hermaphrodite anal depressor muscle. The muscle contractile fibers are labeled with yellow fluorescent protein (YFP)::actin expressed from the *aex-2* promoter. (C–G) DIC images of developing larval males. In (C), cell B.pa is colored in red; in (D–G), the cells B.paa and B.pap are colored in blue and orange, respectively. The outline depicts the approximate shape and location of the anal depressor. (C′–G′) depict cognate fluorescence images of males depicted in (C–G); the anal depressor is labeled with YFP expressed from the *aex-2* promoter. (C) DIC image of an early L3 male. (D) DIC image of a late L3 male tail. (E) DIC image of an early mid-L4 male tail. (F) DIC image of a mid-L4 male tail. (G) DIC image of a late L4 male tail. Bar, 10 μm for all images; dorsal is to the top and anterior is to the left.

contraction. The relaxed sphincter muscle allows fecal waste to pass from the intestine directly into the outside-accessible adult proctodeum (Reiner and Thomas 1995).

Since the adult male has an open cloaca, the anal depressor is no longer needed for gating the orifice. During L4 development, the anal depressor begins remodeling to develop into a muscle used for copulation (Sulston *et al.* 1980; Chen and García 2015). In early L3, the male anal depressor contacts B.pa (a descendant of the B lineage) of the proctodeum (Figure 7B). When B.pa divides at mid-L3 to form B.paa and B.pap, the sex-determination program signals the anal depressor to form a ventral slit between the boundaries of the two B.pa daughters (Figure 7C). The anal depressor region in contact with the B.paa becomes the anterior domain, and the region in contact with B.pap becomes the posterior domain. By the end of L3 development, the dorsal protractor muscle cells (progeny of the male sex myoblasts) have moved adjacent to the anal depressor and signal the defecation muscle to undergo further remodeling. During early L4 development, the anal depressor sarcomere in the anterior domain disassembles and the bilateral anterior regions elongate over the dorsal surface of the sex muscles (Figure 7D). In contrast, the sarcomere in the posterior domain remains intact until after the male hypodermal tail tip completely retracts. When male fan morphogenesis occurs during mid–late-L4 stage, the posterior sarcomere begins to disassemble (Figure 7E). In late L4 stage, the muscle retains some of its old association with the rectal epithelium (B.paa and B.pap), but develops new anterior posterior-oriented sarcomeres aligned with the dorsal protractor muscles (Figure 7F) (Chen and García 2015).

Sex differences in neural circuit connectivity

Some sex differences in the worm nervous system are more subtle than the existence of sex-specific structures. The recent ultrastructural description of the adult male tail connectome, and its comparison with that of the hermaphrodite, revealed numerous instances of apparent sex differences in synaptic connectivity among shared neurons in the tail (Jarrell *et al.* 2012; Oren-Suissa *et al.* 2016). In many cases, the processes of two (or more) shared neurons contact each other in adults of both sexes, but a synapse—detected ultrastructurally and by fluorescent labeling [GRASP (GFP Reconstitutions Across Synaptic Partners) or iBLINC (*in vivo* Biotin Labeling of Intercellular Contacts)]—exists only in one. In a few of these instances, sex-specific synapse formation during larval development accounts for dimorphic connectivity (Oren-Suissa *et al.* 2016). However, a more prominent mechanism for establishing sexual dimorphism is sex-specific synaptic pruning. In this process, synaptic connections between two cells form in larvae of both sexes, but are then sex-specifically eliminated with the transition to adulthood (Oren-Suissa *et al.* 2016). For example, the PHB phasmid sensory neuron synapses onto the interneuron AVA in larval and adult hermaphrodites, contributing to a circuit important for chemosensory aversion (Figure 8). The PHB > AVA connection is also present in larval males, but is sex-specifically pruned during the transition to adulthood. Reciprocally, a different PHB synapse, the PHB > AVG connection, initially forms in both sexes but is specifically pruned in hermaphrodites. Functionally, this repurposed circuitry in the male is thought to be

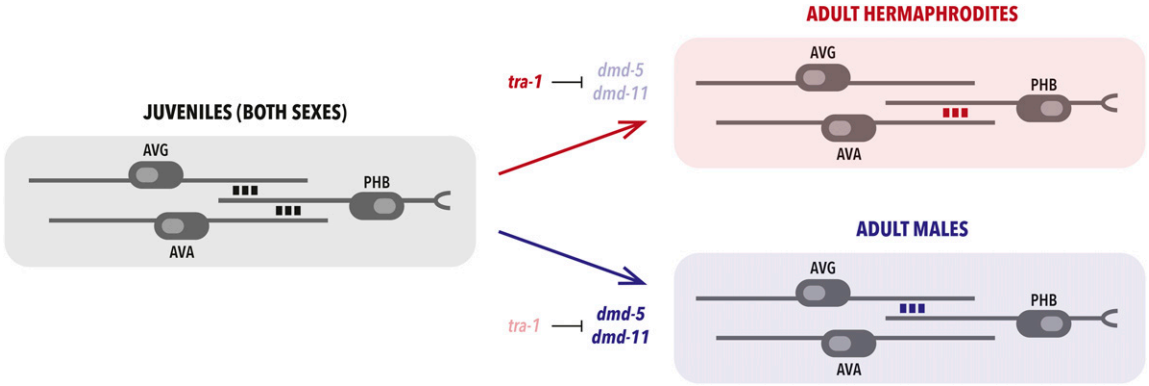

Figure 8 Sex differences in circuit connectivity. PHB, a shared sensory neuron, is one of several tail neurons that participates in sexually dimorphic connectivity. In larvae, PHB connects to several postsynaptic targets, including the shared neurons AVA and AVG. Sex-specific synaptic pruning in late larval development generates differential connectivity: in hermaphrodites, the PHB > AVG connection is selectively degraded, while in males, PHB > AVA is degraded. These events, regulated by *tra-1* and at least two DM domain genes, allow PHB to maintain its function in aversive chemosensory behavior in hermaphrodites, while permitting a role for PHB in promoting copulatory behavior in males.

important for successful mating behavior. *tra-1* appears to mediate these sex-specific events through the action of at least two DM domain transcription factors, *dmd-5* and *dmd-11*, though how these regulate synaptic maintenance is unknown (Oren-Suissa *et al.* 2016).

A related phenomenon is seen in the PHC sensory neuron. In the hermaphrodite, input onto PHC is sparse, but in the adult male, PHC is extensively innervated by male-specific sensory neurons born late in larval development (Jarrell *et al.* 2012; Serrano-Saiz *et al.* 2017a) [note that PHC was incorrectly identified as LUA in Jarrell *et al.* (2012)]. In this case, the DM gene *dmd-3* acts male-specifically in PHC to allow it to take on this "hub neuron" state, acting at least in part by promoting the extension of the PHC axon into the ventral nerve cord (Serrano-Saiz *et al.* 2017a). Similarly, several posterior motor neurons, notably DD06, AS11, and PDB (AS12), are sexually dimorphic, featuring extensive male-specific branching and engagement with the male copulatory circuitry (S. Emmons, personal communication; www.wormwiring.org). In the worm's head, the extent to which synaptic connectivity differs by sex is unclear, but ongoing analysis of the connectome is revealing that this form of dimorphism is likely to be significantly more extensive than has been previously appreciated (S. Emmons, personal communication; www.wormwiring.org).

In addition to these hard-wired anatomical dimorphisms, recent work has found several instances of sexually dimorphic neurotransmitter use. In the shared AIM neuron, glutamate is used as a neurotransmitter in hermaphrodites and in larval males, but upon sexual maturation in males, AIM switches to using acetylcholine (Pereira *et al.* 2015). While the functional significance of this is not known, AIM had been previously shown to be sexually dimorphic, expressing the GPCR *srj-54* only in males (Lee and Portman 2007). Furthermore, analysis of the connectome of the male head has revealed the existence of an AIM–AIB synaptic connection present only in males (S. Emmons, personal communication; www.wormweb.org), suggesting that AIM may be a key focus of sex-specific modification. Several other neurons have also been found to exhibit sex-specific neurotransmitter switching (Serrano-Saiz *et al.* 2017b); understanding how and why this occurs is likely to reveal interesting new insights into *C. elegans* neurobiology.

Physiology and Behavior: Sex-Specific and Sexually Modulated Circuits

Hermaphrodite egg laying

In the hermaphrodite, a sex-specific midbody neuromuscular system regulates the frequency and quantity of egg dispersal (Waggoner *et al.* 1998). In comparison, the male's neuromuscular reproductive anatomy in the tail facilitates copulation attempts with hermaphrodites (Liu and Sternberg 1995). These divergent reproductive behaviors are executed by sex-specific neural control of the M-derived muscles (Sulston *et al.* 1980).

The hermaphrodite egg-laying circuit consists of eight uterine muscles that force eggs out of the uterus and eight vulva muscles that widen the vulval slit (Figure 9A) (see WormAtlas, www.wormatlas.org/hermaphrodite/egglaying%20apparatus/Eggframeset.html). The uterine and vulva muscles are interconnected by gap junctions, suggesting that their contractions are coordinated. The egg-laying muscles' contractile states are regulated by the two hermaphrodite-specific HSN serotonergic motor neurons and six VC cholinergic motor neurons, which innervate the vm2 vulva muscles (Figure 9B) (White *et al.* 1986). These neurons play a central role in regulating the frequency of egg-laying events, presumably to minimize sibling overcrowding in food environments and to ensure that eggs are not laid in stressful situations (Trent 1982; Waggoner *et al.* 1998; Sanders *et al.* 2013; Aprison and Ruvinsky 2014; Fenk and de Bono 2015).

Under abundant food conditions, the hermaphrodite's reproductive behavior alternates between an egg-laying inactive state (retaining eggs in its uterus) and an egg-laying active state (in which single, or a cluster of, eggs are laid)

(Waggoner *et al.* 1998). In the inactive egg-laying state, the outputs of the HSN and VC neurons are attenuated. HSN activity during the inactive state is downmodulated by the heterotrimeric G-protein $G_{\alpha o}$; the G-protein receptors GAR-2, SER-4, EGL-47, and EGL-6; and the hyperpolarizing inward rectifying K^+ channel IRK-1 (Bany *et al.* 2003; Shyn *et al.* 2003; Moresco and Koelle 2004; Dempsey *et al.* 2005; Emtage *et al.* 2012). Similarly, the neuronal outputs of the VCs are attenuated by the hyperpolarizing small conductance calcium-activated K^+ channel KCNL-2 (Chotoo *et al.* 2013). The egg-laying circuitry is also modulated by the number of eggs held in the uterus. The uv1 cells, secretory cells associated with the ventral uterus, are proposed to attenuate egg-laying events until they mechanically sense the accumulation of multiple eggs (Jose *et al.* 2007; Zhang *et al.* 2010).

When the hermaphrodite is in the active egg-laying state, the HSN neurons secrete serotonin, which binds the SER-1 (5-HT_2-like) and SER-7 (5-HT_7-like) GPCRs on the vm2 vulva muscles; these $G_{\alpha q}$-coupled receptors then induce concerted vulval and uterine muscle contractions (Bastiani *et al.* 2003; Carnell *et al.* 2005; Hobson *et al.* 2006; Xiao *et al.* 2006). The stochastic frequency of the HSN firing pattern is partly regulated cell-autonomously by chloride gradients through *kcc-2*-encoded potassium chloride transporters and *clh-3*-encoded chloride channels (Tanis *et al.* 2009; Branicky *et al.* 2014). In parallel, the cholinergic VC neurons sensitize the vulva muscles to HSN secretions and also modulate the frequency of egg-laying events during the active cycle (Waggoner *et al.* 1998; Collins and Koelle 2013). As the hermaphrodite crawls around its environment, the VC neurons are proposed to sense body postural changes and secrete acetylcholine, which hypopolarizes the vm2 vulval muscles. Hyperpolarizing current from the UNC-103, EGL-36, and EGL-2 voltage-gated K^+ channels keep the muscles from prematurely contracting, but also maintains the muscles at a subfiring threshold (Elkes *et al.* 1997; Johnstone *et al.* 1997; Weinshenker *et al.* 1999; Chen *et al.* 2010; Collins and Koelle 2013). When the VC-potentiated muscles are costimulated by the HSN neurons, the vm2 vulva muscles then depolarize and contract. The VC neurons also activate inhibitory $G_{\alpha o}$-coupled GAR-2 receptors on the HSN, suggesting that VC neurons can also fine-tune HSN activity within the active phase (Bany *et al.* 2003; Zhang *et al.* 2010). The coupling of locomotion to egg laying not only facilitates the dispersal of progeny, but also allows the body wall muscle contractions to aid the uterine muscles in egg expulsion (Collins and Koelle 2013; Collins *et al.* 2016).

Although most eggs that are laid are usually produced by internal self-fertilization, the hermaphrodite can also lay eggs produced by cross-fertilization from copulating with males. Copulation has benefits and disadvantages for the hermaphrodite. A selfing hermaphrodite contains enough self-sperm to make a brood of ~270 eggs. In contrast, the outcrossed hermaphrodite gains reproductive and physiological benefits from interacting with the male. It obtains additional sperm to generate many more genetically fit progeny (Morran *et al.* 2009; Carvalho *et al.* 2014). The male seminal fluid also

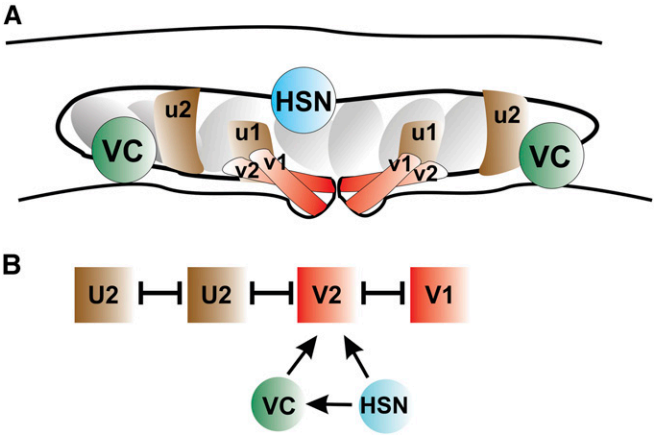

Figure 9 Hermaphrodite egg-laying circuit. (A) Neurons and muscles used in egg-laying behavior. (B) Connectivity of the uterine and vulval muscles with the VC and HSN neurons. Bars represent electrical connections; arrows represent chemical connections.

contains the INS-31 peptide, which might also modulate the hermaphrodite's reproductive physiology (Kim *et al.* 2016). In addition, exposure to low–moderate concentrations of male sex pheromones (ascaroside mixtures; see below) accelerates its reproductive development, promotes reproductive vigor against heat stress, improves sperm guidance toward oocytes, and delays aging-induced decline of germline progenitor cells (Aprison and Ruvinsky 2015, 2016, 2017). However, excessive exposure to males can also reduce the hermaphrodite's longevity. Physical cuticle damage, chronic exposure to external male secretions, and excessive transferred sperm/seminal fluid can induce stress, and alter the hermaphrodite's insulin-like peptide signaling and steroid hormone regulation. The results of these changes are body fragility/shrinkage, reduced osmotic stress resistance, and general behavioral decrepitude. Why excessive male secretions deleteriously alter the hermaphrodite is not known, but this is speculated to be either a form of male-directed population control or sexual conflict to limit the outcrossed hermaphrodite from further copulation (Maures *et al.* 2014; Shi and Murphy 2014; Ting *et al.* 2014; Woodruff *et al.* 2014; Palopoli *et al.* 2015).

Mating signals: ascarosides and other sex pheromones

The ability to find mates is necessary in all sexually reproducing species. In *C. elegans*, the best understood class of compounds that signal the presence of potential mates are the ascarosides, glycosides of the dideoxysugar ascarylose (Ludewig and Schroeder 2013; Chute and Srinivasan 2014). Animals of both sexes secrete numerous varieties of ascarosides, which play roles in communicating population density, developmental, and metabolic state, and other signals important for the survival of *C. elegans* populations. At certain concentrations, several ascarosides promote entry into the long-lived, stress-resistant dauer larva state (Butcher *et al.* 2007; Srinivasan *et al.* 2008). An overlapping set of ascarosides causes sex-specific behavioral responses. In

particular, ascr#2, ascr#3, ascr#4, and ascr#8 elicit strong (and highly concentration-dependent) attraction in males, and can generate repulsion responses in hermaphrodites (Srinivasan *et al.* 2008; Macosko *et al.* 2009; Pungaliya *et al.* 2009; Jang *et al.* 2012; Fenk and de Bono 2017). Some of these, particularly ascr#3, are preferentially produced by hermaphrodites (Izrayelit *et al.* 2012). Several related compounds, icas#3 and icas#9, also attract males (Pungaliya *et al.* 2009). Interestingly, the ascaroside ascr#10 appears to have a reciprocal function: it is preferentially secreted by males and serves to attract hermaphrodites (Izrayelit *et al.* 2012). Because of these sex differences in chemosensory behavior, these molecules are considered to be sex pheromones. However, ascarosides are not the only sex pheromones in *C. elegans*, as several ascaroside-independent attraction behaviors have been identified (White *et al.* 2007; Leighton *et al.* 2014) (see below).

The neural and genetic underpinnings of sexually dimorphic responses to ascarosides are complex and not fully understood. In males, the sex-specific head CEM sensory neurons can detect ascarosides, and seem to do so through an unusual mechanism that allows them to encode dynamic information about ascaroside concentration (Srinivasan *et al.* 2008; Narayan *et al.* 2016). Ablation of the CEMs leads to reduced ascaroside attraction. Shared sensory neurons also have a role in male ascaroside attraction: the ASK neurons promote male attraction (Srinivasan *et al.* 2008; Jang *et al.* 2012). The shared ADL neuron promotes ascaroside avoidance in hermaphrodites, and its activity in males seems to be suppressed for attraction to occur (Jang and Bargmann 2013).

Recent work has found that the shared neuron ADF plays a key role in pheromone-mediated behaviors: the genetic sex of ADF itself is sufficient, regardless of the sexual state of the rest of the body, to determine the valence of an animal's behavioral response to ascaroside pheromones (Fagan *et al.* 2017). Genetic feminization of ADF in males eliminates attraction to an ascaroside mixture and reveals an underlying repulsive drive, while masculinization of ADF in hermaphrodites is sufficient to convert ascaroside repulsion to attraction. Genetic sex modulates ADF by tuning its sensory function, such that it detects ascarosides (predominantly ascr#3) only in males. This sex difference is controlled cell-autonomously by *tra-1*, which acts in part by repressing expression of the DM gene *mab-3* in the hermaphrodite ADF. Importantly, this sex difference is likely to be important for male reproductive fitness, as ADF "maleness" is necessary for males to be able to locate hermaphrodites by detecting the ascarosides they produce (Fagan *et al.* 2017). Although multiple sensory neurons have been implicated in male ascaroside attraction, chemoreceptors necessary for male-specific attraction have not yet been identified. Furthermore, very little is known about hermaphrodite-specific attraction to ascr#10 or other ascarosides (Izrayelit *et al.* 2012).

Learning and memory: sexual conditioning

C. elegans exhibits several interesting forms of experience-dependent plasticity in behavior. In hermaphrodites, one example of this is aversive gustatory conditioning: animals will learn to avoid sodium chloride—to which they are innately attracted—by being exposed to sustained sodium chloride stimulation under starvation conditions (Saeki *et al.* 2001). This effect occurs in both hermaphrodites and males; however, Sakai *et al.* (2013) identified an unexpected sex difference in the nature of this plasticity. When hermaphrodites are present during male starvation conditioning, hermaphrodite cues override the aversive association of sodium chloride with starvation, such that males remain attracted to sodium chloride. This effect, called sexual conditioning, requires males to detect both diffusible and contact-dependent cues from hermaphrodites, and likely provides a mechanism for males to use sodium chloride as a cue to aid mate searching (Sakai *et al.* 2013). Interestingly, the MCMs, recently discovered male-specific head interneurons, are essential for sexual conditioning (Sammut *et al.* 2015). These neurons receive direct and indirect input from the rays and CEMs (Sammut *et al.* 2015), both of which are implicated in detecting sexual conditioning signals (Sakai *et al.* 2013).

Mating signals: hermaphrodite receptivity and mate choice

The hermaphrodite was originally thought to be a passive partner in the mating ritual. However, behaviors of male/female and hermaphroditic *Caenorhabditis* species are influenced by the presence or absence of sperm in the uterus. In the gonochoristic species *C. brenneri* and *C. remanei*, males are attracted to a volatile pheromone produced by unmated females (Chasnov *et al.* 2007). In *C. elegans*, hermaphrodite sperm status influences the receptivity to male advances. Sperm-containing hermaphrodites are twice as likely to sprint away from males during copulation attempts than sperm-depleted hermaphrodites (Garcia *et al.* 2007; Kleemann and Basolo 2007). After making contact, males initiate vulva searching sooner with sperm-depleted hermaphrodites compared with self-sperm-containing hermaphrodites (Kleemann and Basolo 2007), suggesting that males can somehow discern the sperm status of hermaphrodites.

Consistent with this idea, males preferentially mate with older *C. elegans* hermaphrodites (or "sexy cougars") (Morsci *et al.* 2011). These older or spermless hermaphrodites are potent stimuli, and rescue the severe defects in contact response (the first step of male mating behavior; see below) of *lov-1* and *pkd-2* mutant males. The improved response of *pkd-2* males to spermless hermaphrodites is independent of short-chain ascarosides, and suppressed by prior insemination or genetic ablation of the *ceh-18*-dependent sperm-sensing pathway of the hermaphrodite somatic gonad (Morsci *et al.* 2011). Oocyte–somatic gonad communication also regulates the production of a volatile pheromone that is attractive to *C. elegans* males (Leighton *et al.* 2014) and may be the same as the "sexy cougar" cue (Morsci *et al.* 2011; Leighton *et al.* 2014). The cellular and molecular nature of the hermaphrodite-derived pheromone and the male reception/signal transduction pathway remain to be determined.

Mate searching

The mate-searching behavior of the *C. elegans* male involves an exploratory behavior in which the male leaves a food source in search of hermaphrodite partners (Lipton *et al.* 2004). The male must evaluate and prioritize behavioral decisions, based on needs for food and for sexual reproduction, that involve sex-shared behavior (food exploration) and sex-specific behavior (dispersal in search of mates). The goal of male mate searching is to find hermaphrodites on a food source, as hermaphrodites alone do not retain males. For a comprehensive review of male mate-searching behavior, the reader is referred to Barrios (2014).

Regulation of exploration is mediated by at least three internal states: reproductive status in which the germline and DAF-9•DAF-12 nuclear hormone ligand producer/receptor function (Kleemann *et al.* 2008); nutritional state, in which the DAF-2•DAF-16 Insulin and DAF-7 TGFβ pathways play a role (Lipton *et al.* 2004; Hilbert and Kim 2017), perhaps by modulating male food sensitivity (Ryan *et al.* 2014); and neuromodulatory input from the PDF (pigment dispersing factor) and oxytocin/vasopressin-related nematocin neuropeptides (Barrios *et al.* 2012; Garrison *et al.* 2012). Sensory experience also impacts male exploratory behaviors. Males retain the memory of hermaphrodite contact and suppress food-leaving behavior for up to 1 hr after sexual contact (Barrios *et al.* 2008). The contact-based pheromone is found in the cuticle and is independent of short-chain ascarosides produced by *daf-22* (Barrios *et al.* 2008; Gravato-Nobre *et al.* 2011; Barrios 2014).

Recent work has also shown that signaling through the TGFβ-family ligand DAF-7 is important for male exploration. In hermaphrodites, DAF-7 is normally produced specifically by the shared ASI sensory neuron, but in males, the shared neuron ASJ also secretes this ligand (Hilbert and Kim 2017). This sex difference is controlled by the genetic sex of the nervous system itself (though perhaps not by the sex of ASJ) and is important for generating high levels of exploration in males. Ablation of ASJ in males is sufficient to decrease mate-searching behavior. Moreover, the production of DAF-7 by ASJ is regulated by food, coupling feeding status to exploratory behavior (Hilbert and Kim 2017).

The evolutionarily conserved PDF neuropeptide system is a major regulator of male searching behavior (Barrios *et al.* 2012). The ligand PDF-1 and its receptor PDFR-1 stimulate mate-searching behavior in males and not hermaphrodites. *pdfr-1* and *pdf-1* mutant males are leaving assay defective (Las) and also display defects in contact-based response. *pdf-1* and *pdfr-1* act downstream or independently of the DAF-2•DAF-16 pathway, and do not signal nutritional status. *pdf-1* is expressed in the shared interneuron AIM and activates the *pdfr-1* receptor also in shared neurons (URY, PQR, and PHA) to produce male-specific mate-searching behavior. URY is postsynaptic to the CEM neurons that sense secreted pheromones including ascarosides (Srinivasan *et al.* 2008; White and Jorgensen 2012; Narayan *et al.* 2016), hinting at a mechanism by which a sex-shared neuron contributes to a sex-specific behavior (Barrios 2014).

Ray neurons stimulate food-leaving and mate-searching behavior by promoting forward locomotion and repressing reversals signaled by food-sensing amphid channel cilia (Barrios *et al.* 2008). Genetic ablation of the ray RnB sensory neurons or disruption of RnB function by mutations in *pkd-2* or *lov-1* results in the Las phenotype (Barrios *et al.* 2008). Overexpression of PDF-1 in *pkd-2* or *lov-1* mutants partially rescues the Las defect (Barrios *et al.* 2012). Hence, mate-searching behavior is controlled by sex-specific and sex-shared neurons.

The evolutionarily conserved Oxytocin/Vasopressin-related neuropeptide system modulates several male sexual behaviors, including mate searching, mate recognition, and mating behavior (Garrison *et al.* 2012). *C. elegans* NTC-1 nematocin is similar to mammalian vasopressin and oxytocin. The nematocin receptors NTR-1 and NTR-2 are similar to GPCRs related to vertebrate vasopressin and oxytocin receptors. *ntc-1*, *ntr-1*, and *ntr-2* are expressed in the sex-shared and male-specific nervous system, consistent with both sex-specific and sex-shared neural control of male sexual behaviors.

Two other modifications to shared circuits also support male mate-searching behavior. First, to prioritize exploration, well-fed adult males downregulate their attraction to the food-related odorant diacetyl and to bacterial food itself. This results at least in part from the effects of genetic sex (via *tra-1*) in the shared AWA chemosensory neurons (Ryan *et al.* 2014). While adult hermaphrodites express high levels of the diacetyl chemoreceptor *odr-10*, expression of this gene is low in adult males, resulting in decreased attraction to diacetyl and to food (Lee and Portman 2007; Ryan *et al.* 2014). Genetically sex-reversing AWA in males, or overexpressing *odr-10*, is sufficient to drive increased food attraction and to suppress food leaving. Interestingly, some of the effects of food deprivation on male behavior also seem to be mediated through *odr-10*, as starvation increases *odr-10* expression and food attraction while suppressing mate searching (Lipton *et al.* 2004; Ryan *et al.* 2014).

Another adaptation that promotes male mate searching is the modulation of locomotion itself: males exhibit deeper body bends, and execute these body bends more frequently, than hermaphrodites (Mowrey *et al.* 2014). The net result of this is that males can travel faster and can likely navigate through challenging terrain more effectively than hermaphrodites. These sex differences arise both through changes in the properties of male body wall muscles, as well as changes in sensory physiology that, via unknown mechanisms, promote high body-bend rates in males (Mowrey *et al.* 2014).

Male copulatory behavior

Once a mate is located, the multistep process of copulation, entailing the interactions of multiple sex-specific muscles and neurons, ensues. Copulation can be reduced into a series of stereotyped sensorimotor subbehaviors (Figure 10A): contact response, where the male positions its tail against the hermaphrodite cuticle; vulva-searching behavior, where it moves

backward along the hermaphrodite scanning for the vulva; turning, where it reorients itself to the other side of the hermaphrodite; vulva sensing and spicule intromission attempts, where it senses the vulva, stops backward locomotion, and repetitively attempts to breach the vulva with its copulatory spicules; and spicule penetration and sperm transfer, where it fully inserts its copulatory spicules, ejaculates, and finally swims away. These subbehaviors generally follow a stepwise progression, beginning with contact response and finishing with sperm transfer; however, the male can repeat subbehaviors and shift between any of these motor outputs (Figure 10B) (Liu and Sternberg 1995).

The nine bilateral pairs of sensory rays contained in the male fan play continuous and overlapping roles in executing contact-response, vulva-searching, and turning behavior. Each ray pair contains the dendrites of the A- and B-type sensory neurons. The A- and B-type neurons have distinct cilia morphology, as well as different neurotransmitter content and synaptic connections with multiple partners. The sensory ending of an A-type neuron is more recessed in the sensory ray. In addition, the sensory ending has a long striated rootlet, but no obviously discernable basal body. In contrast, the B-type neuron projects its sensory dendrite to the tip of the ray opening; a distinct basal body is also associated with its ciliated ending (Sulston *et al.* 1980). Other male-specific sensory organs or sensilla display the same organization as ray sensilla, with A- and B-type sensory neurons. In the hook and cephalic sensilla, the HOB and CEM neurons display morphological and molecular attributes similar to the ray B-type neurons (see below). The rays are numbered 1 through 9 (anterior to posterior), reflecting their specialized characteristics along this axis. Except for ray pair 6, the tips of the rays are open to the environment, so that neuronal sensory endings are exposed at the dorsal, ventral, or marginal sides of the fan. The ray pairs have redundant functions in sensing chemical and mechanical characteristics of the hermaphrodite cuticle. Any three ray pairs are sufficient to elicit contact response, vulva searching behavior, and turning at some efficiency (Liu and Sternberg 1995). However, differences in downstream connections make individual ray pairs and neuronal types better at facilitating specific behavioral substeps. For example, males that lack functional B-type ray neurons, either through laser ablation or through mutation of B neuron-specific cilia genes, have difficulty sensing the hermaphrodite upon physical contact, but eventually can execute vulva-searching behavior similar to that of normal males (Barr and Sternberg 1999; Barr *et al.* 2001; Miller and Portman 2010). In contrast, laser-operated males that lack ray A-type sensory neurons have difficulty in executing contact-response and vulva-searching behavior (Koo *et al.* 2011).

The polycystins LOV-1 and PKD-2: connecting male behavior to sensory cilia

The *C. elegans* polycystins LOV-1 and PKD-2 are required for male mating behavior, act in the same genetic pathway, and localize to sensory cilia (Barr and Sternberg 1999; Barr *et al.* 2001). This discovery was one of the first links between cilia and human disease (the polycystins PKD1 and PKD2 are mutated in human autosomal dominant polycystic kidney disease) (Watnick and Germino 2003). The *C. elegans* polycystins LOV-1 and PKD-2, as well as the "coexpressed with polycystin" genes *cwp-1* through *cwp-5* (Portman and Emmons 2004), are expressed and act in 21 male-specific sensory neurons. The CEM neurons have a role in chemotaxis to hermaphrodites (Chasnov *et al.* 2007; Srinivasan *et al.* 2008), while the RnB neurons are important for both non-contact-based mate-searching behavior (Barrios *et al.* 2008) and contact-based response behavior (Koo *et al.* 2011), and the HOB neuron is needed for vulva-location behavior (Liu and Sternberg 1995). The male cephalic, ray, and hook sensilla all share a similar anatomical arrangement and ultrastructure, with a putative chemosensory B-type ciliated sensory neuron whose tip protrudes to the environment and an A-type ciliated sensory neurons whose cilium is embedded in the cuticle surrounded by glial support cells (socket and sheath cells for the cephalic and hook sensilla) (Ward *et al.* 1975; Sulston *et al.* 1980). Herein we refer to the CEM, RnB, and HOB neurons as the 21 male-specific B-type neurons. *lov-1* and *pkd-2* mutant males are defective in mate searching, response, and vulva location (Barr and Sternberg 1999; Barr *et al.* 2001; Barrios *et al.* 2008).

PKD-2 ciliary localization is regulated by cell-specific and general mechanisms (Bae *et al.* 2006), including genes that affect microtubule-based motor transport, microtubule stability, membrane dynamics, and post-translational modifications [reviewed in O'Hagan *et al.* (2014)]. In addition to its role in PKD-2 ciliary localization, the kinesin-3 *klp-6* is required for response and vulva-location behaviors, and is a negative regulator of mate searching (Peden and Barr 2005; Maguire *et al.* 2015). *klp-6* is also important for the environmental release of ciliary extracellular vesicles (EVs) (Wang *et al.* 2014). Consistent with these functions, *klp-6* is expressed in the 21 male-specific B-type neurons and six sex-shared IL2 neurons. In the hermaphrodite, the six inner labial sensilla are similar to the cephalic, ray, and hook sensilla, and possess A-type IL2 and B-type IL2 neurons (Perkins *et al.* 1986).

The IL2 and the male-specific B-type neurons are unique in *C. elegans* in that they shed ciliary extracellular vesicles (Wang *et al.* 2014), are members of sensilla or sensory organs composed of pairs of A-type (cilium not exposed to environment) and B-type (cilium exposed to environment) neurons, have cilia that protrude through cuticular pores (Ward *et al.* 1975) (Figure 11) (www.wormatlas.org/hermaphrodite/neuronalsupport/mainframe.htm), and express the DAF-19m transcription factor. DAF-19m (for function in mating) is an isoform of the sole RFX (regulatory factor X)-type transcription factor in *C. elegans*, which is an evolutionarily conserved master regulator of ciliogenesis genes (Swoboda *et al.* 2000; Wang *et al.* 2010).

Cilia are organelles with a microtubule core, called the axoneme, that exhibit a conserved architecture of nine outer

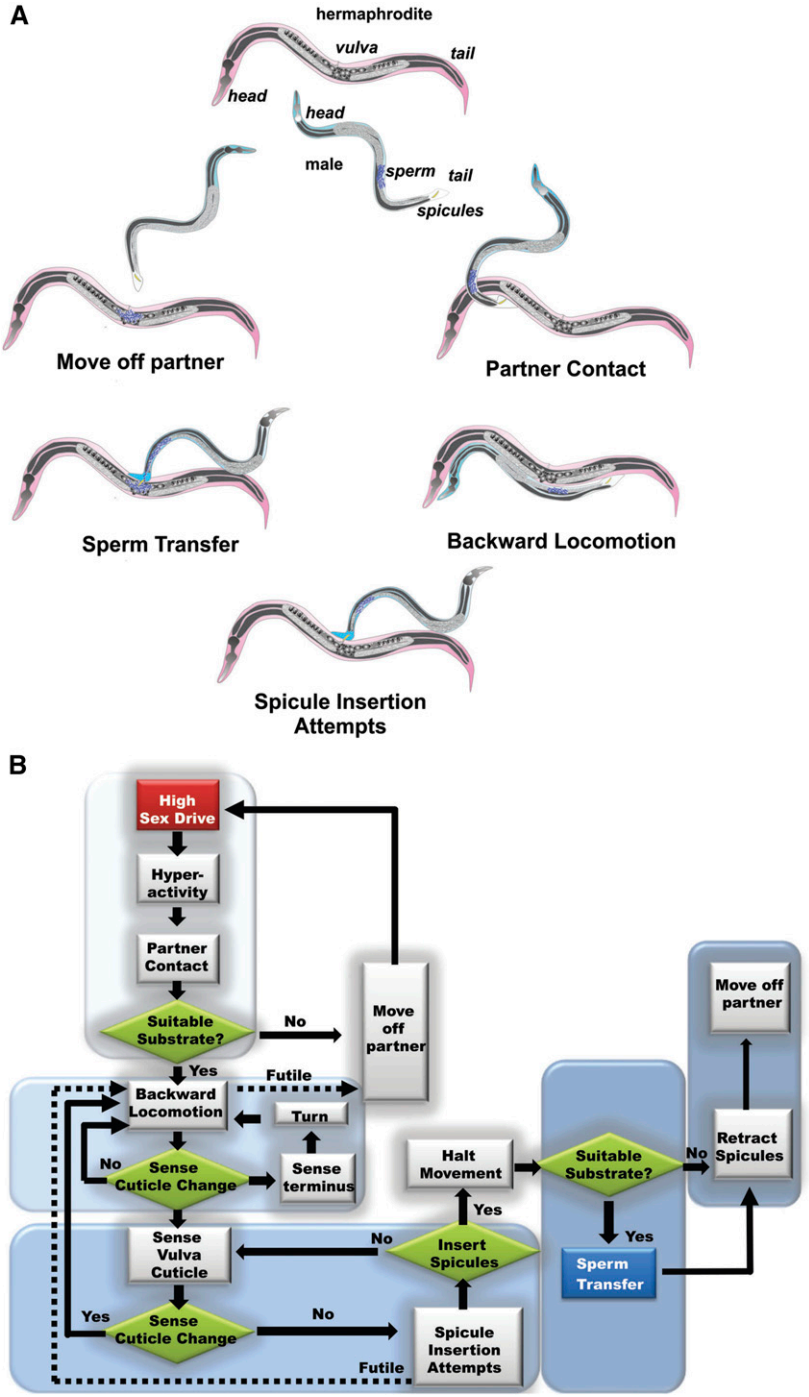

Figure 10 Male copulation behavior. (A) Cartoon of the different steps performed by the male during copulation. (B) Decision flow diagram of the different motor behaviors used during copulation. Dashed arrows represent outcomes that may occur if a male performs a particular motor routine for too long.

doublet microtubules with a variable number of inner singlets (Rosenbaum and Witman 2002; Fisch and Dupuis-Williams 2011). Axonemal variations may arise in the relative length of each region, and the presence or absence of the distal segment, where A-tubule singlets extend and B-tubules terminate (Fisch and Dupuis-Williams 2011). *C. elegans* possess variant 9 + 0 cilia whose ultrastructures can be simultaneously analyzed using transmission electron microscopy (TEM) and electron tomography (Perkins *et al.* 1986; Doroquez *et al.* 2014). Variant 9 + 0 cilia are not nematode-specific oddities. Variations from the "typical" 9 + 2 and 9 + 0 doublet structure may be more common than appreciated, largely due to technical difficulties associated with serial section TEM of mammalian cilia.

CEM cilia display an ultrastructural specialization in which nine microtubule doublets splay into distinct A-tubules and B-tubules to generate 18 singlets in middle regions of the axoneme, but remain joined in distal and proximal regions (Silva *et al.* 2017). Flagella of human and rat spermatozoa also display A- and B-tubule singlets extending from microtubule doublets (Woolley and Nickels 1985; Afzelius *et al.* 1995), hinting that a conserved but unidentified mechanism

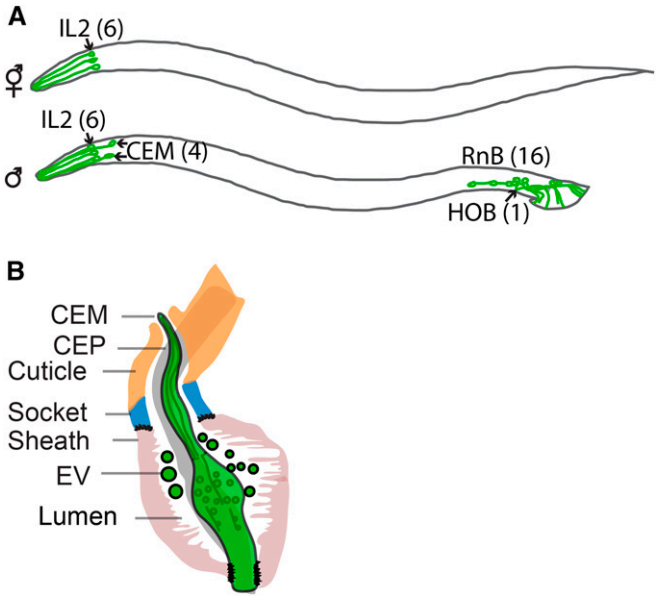

Figure 11 Extracellular vesicles (EVs). (A) EV-releasing neurons in the hermaphrodite and male. Six sex-shared IL2 neurons shed and release EVs. In the male, four CEM neurons in the head, and 16 RnB neurons (not R6B) and the HOB neuron in the tail, shed and release EVs. (B) Cartoon representation of electron microscopy tomogram of cephalic sensillum that is comprised of the CEM and CEP cilia surrounded by the glial socket and sheath cells. CEM-derived EVs are shed at the ciliary base and accumulate in the lumen formed by the glia. The CEM cilium protrudes through a cuticular pore into the environment. Image reproduced from Wang *et al.* (2014).

generates this structure. Tubulin isotypes and tubulin-modifying enzymes are implicated in CEM ciliary specialization. α-tubulin TBA-6, the CCPP-1 deglutamylase, and TTLL-11 glutamylase regulate CEM structure, intraflagellar transport, and function (Hurd *et al.* 2010; O'Hagan *et al.* 2017; Silva *et al.* 2017).

Regulation of male locomotion and posture during copulation

During the contact response and vulva-searching steps of copulation, the male uses its ray neurons and sex-specific interneurons to alter the function of sex-common locomotor circuitry. Insights from the male connectome, in conjunction with experimental laser ablation of male-specific sensory and interneurons, suggest a plausible circuit for contact-response behavior (Jarrell *et al.* 2012; Sherlekar and Lints 2014). When the rays contact the hermaphrodite, the connectome suggests that they could stimulate the male-specific EF interneurons (progeny of the F and U blast cells). In turn, the EF neurons might inhibit the sex-shared AVB command interneuron, thus attenuating the male's forward movement. The male then executes either a ventral or dorsal coil (depending on what part of its fan contacts the hermaphrodite cuticle), pressing its ventral side against its mate. This appositional posture requires the coordinated cholinergic-, serotonergic-, GABAergic-, and dopaminergic-induced contractions of the posterior sex-shared dorsal and ventral body wall muscle, as well as the male-specific diagonal muscles. The ray neurons then stimulate the male-specific cholinergic PVY and PVX interneurons, which in turn activate *acr-18*-, *acr-16*-, and *unc-29*-encoded ionotropic acetylcholine receptors on the sex-shared, backward locomotion AVA command interneuron. As a consequence, the male moves backward along the hermaphrodite until it approaches the terminus of the hermaphrodite or senses the vulva (Sherlekar *et al.* 2013).

Turning behavior consists of multiple steps: reducing the velocity of backward locomotion upon approaching the end of the hermaphrodite, initiating a ventral tail arch before reaching the end of the hermaphrodite, sliding the arched tail to the other side of the hermaphrodite's body, and propagating a ventral contraction–relaxation wave along its body while continuing to back along the hermaphrodite (Liu *et al.* 2007). Effective turning requires the posterior rays (ray pairs 7, 8, and 9), the male-specific serotonergic CP neurons, the male-specific M-cell-derived oblique and diagonal muscles, the sex-shared ALM, AVM, PLM, and PVM mechanosensory neurons, and body wall muscles (Liu *et al.* 2007). The posterior rays may contribute to the initial sensing of curvature change at the ends of the hermaphrodite (Liu and Sternberg 1995). Along with the rays, the serotonergic CP motor neurons regulate the coordinated contraction–relaxation kinetics of the male-specific oblique and diagonal sex muscles and sex-shared body wall muscles (Loer and Kenyon 1993; Whittaker and Sternberg 2009; Jarrell *et al.* 2012). Interestingly, the sex-shared ALM, AVM, PLM, and PVM mechanosensory neurons use FMRFamide-like neuropeptides (FLP-8, FLP-10, FLP-12, and FLP-20) to signal the male to slide its arched tail to the other side of the hermaphrodite's body and continue backward locomotion. Normally these neurons' function is to signal the locomotion command interneurons to alter the male and hermaphrodite's trajectory upon mechanical stimulation. However, mutant males that are compromised for these mechanosensory neurons will not make a turn. A mutant will hesitate at the end of the hermaphrodite, and move back and forth while repetitively arching its tail (Liu *et al.* 2007). Males containing a mutation in the diagonal muscle-expressed SHL-1 voltage-gated potassium channel also show a similar phenotype (Chen *et al.* 2015); in this case, the hyperexcited diagonal muscles keep the mutant males from following through with the turn. During the initiation of normal turning, the sex-shared mechanosensory neurons might sense the initial postural changes in the arched tail, and subsequently modulate the relaxation kinetics of the diagonal and body wall muscles, while reinforcing the backward locomotion circuit.

Similar to the execution of turning behavior, when the male approaches the vulva, it also alters its backward locomotor velocity and stops when it contacts the cuticle of the vulval lips. This vulva-location behavior requires the bilateral postcloacal sensilla neurons PCA, PCB, and PCC (progeny of the Y.p blast cell), and the hook sensillum neurons HOA and HOB (progeny of P10.p). The sensory endings of these neurons are located at

the immediate fore and rear of the cloacal opening. The hook neurons are proposed to sense chemical and/or physical features associated with the vulva, such as chemicals emitted from the genital orifice or cuticle changes near the vulva. The postcloacal sensilla neurons are proposed to detect distinct local features of the vulva, such as the vulva cuticle, or alternatively sense an abrupt cuticle transition between the hermaphrodite body and the vulval lips (Liu and Sternberg 1995). The molecular composition of the HOB cilium is similar to the those of the ray B-type neurons. Cilia mutations that disrupt B-type function for contact response behavior also disrupt the HOB's function in sensing the vulva (Barr and Sternberg 1999; Barr *et al.* 2001; Peden and Barr 2005; Bae *et al.* 2009). In contrast, the sensory cilia of HOA and the postcloacal sensilla neurons have not been extensively studied. The primary postsynaptic target of HOA is the sex-shared AVG interneuron; recent work has shown that complex interactions between multiple cell-adhesion proteins are necessary to establish the specificity of this connection during male tail development (Kim and Emmons 2017).

Cuticular cues for response and vulva location

Males of the Australian wild isolate AB2, but not most *C. elegans* strains, deposit copulatory plugs on other males' excretory pores (Gems and Riddle 2000b). Male–male plugging is also observed in *C. briggsae* in a strain-specific manner [noted in methods section of Garcia *et al.* (2007)]. The genetic basis of this male–male behavior is a loss-of-function mutation in the *plep-1* gene (Noble *et al.* 2015). *plep-1* is expressed in the excretory cell of both sexes, and its loss may cause the male excretory pore to resemble or produce cues similar to the hermaphrodite's vulva (Noble *et al.* 2015).

The cuticle on the hermaphrodite's vulva expresses distinct lectin-binding surface antigens that bind wheat germ agglutinin (Link *et al.* 1988), suggesting that these surface cues may allow the male to distinguish the vulva from other areas of the body (M. Barr and P.W. Sternberg, unpublished data). Consistent with a role for hermaphrodite surface cues in male mating behavior, males display impaired contact-based response to contact with hermaphrodite mutants defective in genes regulating surface glycosylation (*bus-2*, *bus-4*, *bus-8*, *bus-12*, *bus-17*, and *srf-3*) (Gravato-Nobre *et al.* 2011).

Spicule insertion and sperm transfer

Upon contact with the vulva, secretions from the postcloacal sensilla neurons stimulate acetylcholine and glutamate ionotropic receptors on the cloaca-associated oblique and gubernaculum muscles. The postcloacal sensilla neurons also make synaptic connections with each other, suggesting that they amplify each other's output. The contractions of the oblique and gubernaculum muscles cause the cloacal region to apply force against the vulval lips, stabilizing the male tail over the hermaphrodite's genital orifice (Liu *et al.* 2011). As long as the male cloaca remains positioned over the vulva orifice, the male will repetitively attempt to insert spicules. However, if the cloacal sensilla sense that the cloaca has shifted off the vulva cuticle to the hermaphrodite's body cuticle, then it will readjust its local position or reinitiate backward locomotion to locate the vulva again (Figure 10A).

The gubernaculum muscles are electrically connected to the remodeled anal depressor, which in turn is electrically coupled to the spicule protractor muscles (Jarrell *et al.* 2012). When the cloacal region contacts the vulva, the electrically-coupled protractor muscles undergo rapid (7–11 Hz) contractions, causing the attached spicules to prod against the vulval slit. These rhythmic high-frequency contractions require intracellular calcium mobilization through the *unc-68*-encoded sarcoplasmic ryanodine receptor (Garcia *et al.* 2001; Garcia and Sternberg 2003).

Unless the hermaphrodite dislodges the male from its genital area, the male will attempt to intromit spicules until it self-terminates the spicule prodding behavior or breaches the hermaphrodite orifice. The dopamine-secreting ray neurons are used to terminate unproductive spicule insertion attempts. If the male takes too long to intromit its spicules, prolonged activity of the postcloacal sensilla neurons stimulates the dopaminergic ray neurons (A-type neurons of ray pair 5, 7, and 9). Secreted dopamine then activates inhibitory *dop-2* and *dop-3*-encoded D2-like G-protein receptors on the interconnected postcloacal sensilla neurons and the spicule protractor muscles. The secreted dopamine also promotes hyperpolarizing electrical coupling between the postcloacal sensilla and the hook sensillum neurons. Coattenuation of both vulva sensing organs allows the male to move off an impenetrable vulva (Correa *et al.* 2015).

The spicule protractor muscles and the remodeled anal depressor are directly innervated by the cholinergic SPC motor neurons (descendants of the B.a blast cell). These neurons also contain a sensory ending that is attached to the base of the spicule. If the male's spicules penetrate the vulva before dopamine signaling terminates intromission attempts, the SPC sensory ending presumably detects a change in the spicule position and consequently triggers the sustained protractor muscle contraction. In contrast to the 7–11 Hz contractions that occur during intromission attempts, sustained protractor contraction requires extracellular calcium influx through the *egl-19*-encoded voltage-gated calcium channel (Garcia *et al.* 2001). Concurrent with sustained penetration of the spicules, the SPC motor neurons and the male-specific CA ventral cord neurons also initiate sperm transfer (Schindelman *et al.* 2006).

Similar to preceding subbehaviors used in male copulation, sperm transfer is also a regulated multistep process. Prior to sperm transfer, spermatids are contained in the seminal vesicle of the somatic gonad. Immediately after the spicules penetrate the vulva, seminal fluid (containing sperm-activation factors such as the TRY-5 protease) is secreted from the vas deferens and valve (Smith and Stanfield 2011). These factors travel through the cloaca and into the hermaphrodite vulva and uterus. The seminal valve then dilates, allowing sperm cells to move from the seminal vesicle down the vas deferens. Sperm collects in the vas deferens until the spicule sensory

neurons trigger the sperm to be released from the vas deferens/cloacal region into the hermaphrodite. Each copulatory spicule encases the dendrites of the SPD and SPV sensory neurons. The ciliated endings of these neurons are exposed through an opening at the spicule tips. Presumably, these neurons sense physical or chemical factors that are enriched in the vulval and uterine region. After the spicules penetrate the vulval channel, the SPD and SPV sensory neurons signal the SPC motor neurons, and the PCA and PCB postcloacal sensilla neurons, to induce gubernacular erector, retractor, and anal depressor muscle contraction (Liu *et al.* 2011; LeBoeuf *et al.* 2014). Shortening of these muscles pulls the proctodeum posteriorly and readjusts the inserted spicules, which widens the conduit between the vas deferens and the cloacal opening (LeBeouf and Garcia 2017). Sperm and additional seminal fluid then drains into the hermaphrodite. After the male transfers sperm, it retracts its spicules back into the proctodeum. If the male contains a functional *plg-1* gene, it will then deposit a mucous copulatory plug over the vulval slit (Hodgkin and Doniach 1997; Palopoli *et al.* 2008). As the male releases sperm and seminal fluid into the hermaphrodite, the glia-like spicule socket cells secrete dopamine, which subsequently dampens the activity of the male's reproductive circuitry.

After the postcoital male transfers sperm and produces the plug, it sluggishly moves away from the hermaphrodite. Although the male's sensory rays, postcloacal sensilla, hook sensillum, and male-specific and sex-shared amphid and phasmid sensilla are in the immediate vicinity of its mated partner, these sensory organs are refractive to physical and chemical cues from the hermaphrodite. The reduced activity of the male's reproductive circuitry is partly due to dopamine signals that are released during ejaculation; however, other yet-to-be-determined transmitters and neuropeptides are likely also involved in downregulating the male's sex drive. The male's low sex drive, defined by a refractory period to hermaphrodite cues, can last from 5 to 30 min. Interestingly, exceptional males, which immediately regain their sex drive and insert their spicules into a new mate 3–9 min after their previous mating bout, do not sire progeny. This suggests that the male requires at least 9 min to regenerate a store of sperm. The refractive period might indirectly keep males from attempting to transfer sperm into the same partner, or expending energy copulating with hermaphrodites until fertile gametes are regenerated (LeBoeuf *et al.* 2014).

Male-to-female signaling

Gonochoristic (male–female) *Caenorhabditis* species require successful matings for species propagation and employ several signals that androdioecious (male–hermaphrodite) *Caenorhabditis* species do not possess: hermaphrodites have lost behavioral responses to males (Garcia *et al.* 2007; Kleemann and Basolo 2007) and males display reduced competitive abilities (Palopoli *et al.* 2008; Ting *et al.* 2014). Gonochoristic *Caenorhabditis* females behave differently than androdioecious *Caenorhabditis* hermaphrodites during mating due to a soporific cue produced by males (Garcia *et al.* 2007). Contact between the *C. remanei* female vulva and male tail induces female inactivity: the female simultaneously stops locomotion and defecation, and reduces pharyngeal pumping behavior. *C. briggsae*, but not *C. elegans*, males also produce the soporific cue to immobilize C. *remanei* females during copulation. *C. elegans* and *C. briggsae* hermaphrodites are not affected and have lost the ability to respond to the soporific signal.

Gonochorisitic *C. remanei* males also aerosolize compound(s) only during coitus that attract *C. remanei* females and may act as species-specific mating pheromones (Markert and Garcia 2013). The gonad is required for the male's ability to sedate its mate and for attracting additional females during copulation (Garcia *et al.* 2007; Markert and Garcia 2013). The nature of the soporific and coital cues is not known.

EVs and interorganismal signaling

Several *C. elegans* neuron types, most of which are male-specific, release EVs that function in animal–animal communication and may be important for mating-related behaviors (Wang *et al.* 2014). EVs are tiny membrane-bound mediators of cell–cell communication. Exosomes and ectosomes are two main types of EVs that differ in size and origin (Mathivanan *et al.* 2010; Raposo and Stoorvogel 2013). Exosomes are 40–100 nm in diameter and are released by fusing of the multivesicular body with the plasma membrane. Ectosomes (also known as microvesicles) are 100–1000 nm in diameter and are generated by pinching off of the plasma membrane. EVs carry specific lipid, protein, and nucleic acid cargoes that can be transferred between donor and recipient cells without requiring direct contact. Functions of EVs include protein disposal and cell–cell communication. In mammals, EVs play important roles in normal physiology, and pathological roles in cancer, infectious disease, and neurodegenerative disorders (Cocucci *et al.* 2009; Gyorgy *et al.* 2011). Most functional studies have been done with purified EVs on cultured cells, hence the *in vivo* roles of EVs are largely unknown.

In *C. elegans* and *Drosophila*, EVs play important *in vivo* roles in development and behavior [reviewed by Wang and Barr (2016), Beer and Wehman (2017)]. In the *C. elegans* embryo, microvesicles regulate the cell adhesion necessary for morphogenesis and gastrulation: overproduction of EVs in a phosphoethanolamine flippase *tat-5* mutant causes defects in these processes and resultant embryonic lethality (Wehman *et al.* 2011). In *C. elegans* larvae and adults, seam cells release exosomes containing Hedgehog-related proteins that are required for alae formation (Liegeois *et al.* 2006). In the adult *C. elegans* male, EVs promote male-specific mating-related behaviors.

In *Chlamydomonas*, *C. elegans*, and mammals, EVs are closely associated with cilia, suggesting that cilia may be essential in EV-mediated communication as both senders and receivers [reviewed in Wood and Rosenbaum (2015), Wang and Barr (2016)]. In both males and hermaphrodites, the six IL2 ciliated sensory neurons shed and release EVs (Figure 11A). The male possesses an additional 21 ciliated

sensory neurons that shed and release EVs: the CEMs in the head and, in the tail, the hook HOB neuron and the ray RnB neurons (with the exception of ray 6, which is not exposed to the environment) (Figure 11A) (Wang *et al.* 2014). These neurons shed and release GFP-tagged LOV-1, PKD-2, CWP-1, degenerin channel ASIC-2, myristoylated coiled-coil protein CIL-7, and tubulin glutamylase TTLL-11 from ciliary-derived EVs into the environment from exposed ciliated sensory endings (Figure 12) (Wang *et al.* 2014, 2015; Maguire *et al.* 2015; O'Hagan *et al.* 2017).

EV shedding appears to be selective for specific cargo and cell types: numerous other GFP reporter proteins, including soluble GFP, tubulin, intraflagellar transport polypeptides and motors, KLP-6, or ciliary receptors, are not secreted in EVs from IL2 or male-specific B-type neurons or amphid channel cilia (Wang *et al.* 2014). CEM neurons shed EVs at the ciliary base into the lumen formed by glial support cells (Figure 11B) and EVs may be released through pores in the animal's cuticle into the environment (Figure 12), where they can signal to other animals (Wang *et al.* 2014).

EVs isolated from adult *him-5* cultures via ultracentrifugation promote male-specific behaviors in a cargo-dependent manner (Wang *et al.* 2014). *klp-6* EVs contain endogenous LOV-1 but not PKD-2::GFP, indicating that *klp-6* may play a role in EV cargo selection. EVs isolated from wild-type and *klp-6* animals do not act as long-range chemoattractants, but do promote changes in male locomotor behaviors. Wild-type but not *klp-6* EVs trigger male-specific tail-chasing behavior. EV-triggered reversals and tail-chasing behavior may represent components of a male behavioral repertoire aimed at optimizing reproductive success with self-fertilizing hermaphrodites.

Transcriptional profiling of FACS-purified *klp-6*::*gfp*-expressing EV-releasing neurons from mixed sex adults identified 335 significantly overrepresented genes, of which 61 were validated by GFP reporters (Wang *et al.* 2015). This transcriptional profiling of the EV-releasing neurons and forward genetic screens identified potential core regulators of EV biogenesis/function as well as cell-specific components (Bae and Barr 2008; Maguire *et al.* 2015; Wang *et al.* 2015). Whether EVs play additional roles in *C. elegans* behaviors is an interesting avenue of exploration, as is the question of molecular and functional differences between hermaphrodite-derived *vs.* male-derived EVs.

A connection between mating behavior and innate immune response?

A large proportion of the EV-releasing neuronal signature genes contain domains associated with adhesion and innate immune response (Wang *et al.* 2015). Some key players in innate immunity are found among these genes, as well as effector genes, including C-type lectins, detoxifying genes, stress resistance genes, and antimicrobial peptides. The most highly upregulated antimicrobial peptide F14D7.11 localizes to IL2 and male-specific B-type neuronal cell bodies and cilia, and is also found in environmentally released EVs. Interestingly, human urinary exosomes are also enriched for innate

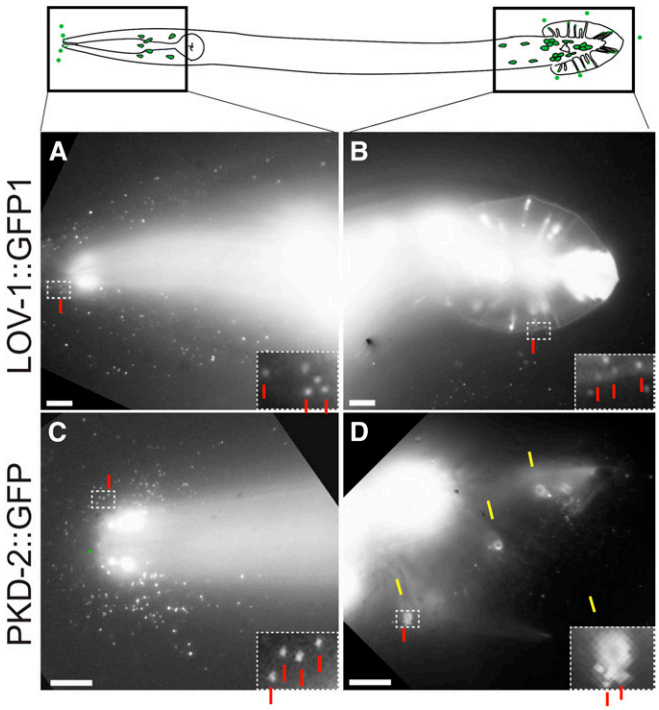

Figure 12 GFP-extracellular vesicle (EV) release from head and tail. GFP-tagged LOV-1 (A and B) and PKD-2 (C and D) are shed and released from ciliated sensory neurons in the head (CEMs) and tail (RnBs and HOB). Boxed insets with red arrows point out GFP-labeled EVs. In (D), EVs accumulate around the cuticular openings of ray sensilla, yellow arrows. Bar, 5 μm. Image reproduced from Wang *et al.* (2014).

immune proteins, including antimicrobial proteins and peptides that have bactericidal properties (Hiemstra *et al.* 2014).

The stress-activated p38 MAPK *pmk-1* is an EV-releasing neuronal signature gene and is required for EV biogenesis (Wang *et al.* 2015). *pmk-1* mutant males are defective in response and vulva-location, but not mate-searching, behavior. Upstream components of the innate immune MAPK cascade *tir-1*, *nsy-1*, and *sek-1* are not required for EV biogenesis or male mating behavior, indicating that *pmk-1* function is independent of this pathway.

Two tumor necrosis receptor-associated factor (TRAF) homologs, *trf-1* and *trf-2*, are coexpressed with the polycystins in the 21 male-specific EV-releasing neurons. *trf-1* and *trf-2* are required for mate searching, contact-based response, and location of vulva behaviors, but are not necessary for EV biogenesis, shedding, or release (Wang *et al.* 2015). In mammals and *Drosophila*, Toll-like receptors and TNF signaling are important for antimicrobial defense. In *C. elegans*, the single Toll-like receptor TOL-1, TRF-1, and downstream signaling molecules do not play a major role in innate immunity (Pujol *et al.* 2001). Genetic analysis indicates that the polycystins and TRAFs act in the same pathway (Wang *et al.* 2015).

An intriguing hypothesis is that *C. elegans* males coopted genes involved in immunity for mating purposes. Alternatively, these findings may reflect sex differences in the

function of the *C. elegans* innate immune system. *C. elegans* males are more resistant to *Pseudomonas aureginosa* and yeast *Cryptococcus neoformans* (Tan *et al.* 1999; van den Berg *et al.* 2006). In the presence of *Bacillus thuringiensis*, male survival is lower than that of hermaphrodites (Masri *et al.* 2013). In these conditions, male sexual activity is reduced and male escape is increased in mixed-sex groups, with almost 100% of males escaping (Masri *et al.* 2013). This is in contrast to male behavior on nonpathogenic *Escherichia coli* OP50, where hermaphrodites retain males on bacterial lawns. An interesting possibility is that males evolved mechanisms to select healthy mating partners.

Male life span: stress responses, aging, and mating decline

C. elegans males and hermaphrodites also show differences in stress resistance and life span. In the laboratory, *C. elegans* life span is culture condition-dependent. Nonetheless, Bristol N2 males kept isolated from one another can have a median life span that exceeds that of hermaphrodites by ~15–20%. This sex bias in longevity has also been observed in other *C. elegans* strains and other nematode species (Gems and Riddle 2000a; McCulloch and Gems 2003). Stress responses have been observed to differ in a similar direction: adult males show enhanced resistance to long-term anoxia compared to hermaphrodites, and this effect occurs through a *daf-16*-independent mechanism (Mendenhall *et al.* 2009).

In contrast, if a male continuously copulates with hermaphrodites, then the male's life span decreases by ~35%. The male's germline is involved in postmating death; when germline proliferation is blocked by either chemical treatment or genetic mutation, postmating death does not occur. Chronic mating also induces abnormal expression of vitellogenins (yolk protein precursors that should only be expressed in the hermaphrodite) in the anterior intestine of aged males. The abnormal intestinal expression of vitellogenins is also dependent on the male germline. In aged hermaphrodites, overexpression of vitellogenins can reduce hermaphrodite life span, and ectopic vitellogenin expression might have a similar effect on the male (Seah *et al.* 2016; Shi *et al.* 2017).

Similar to male–hermaphrodite interactions, chronic exposure to male ascaroside pheromones will also reduce the male's life span. When *C. elegans* males are cultured in groups, even as few as two animals, the male's life span will also decrease by ~20–50%. Male-conditioned media from wild-type males, but not from pheromone biosynthesis mutants, is also effective at reducing the life span of isolated males. The germline is involved in male pheromone killing; however, its mode of action is distinct from mating-induced death. This is evident through transcriptional profiling, as well as fat and glycogen differences between aged males that continuously mated and males that are chronically exposed to pheromone (Gems and Riddle 2000a,b; Shi *et al.* 2017)

Regardless of the effects on male life span caused by rearing conditions, an N2 male's ability to copulate with a moving hermaphrodite declines soon after reaching adulthood and long before its death (Guo *et al.* 2012). Age-related male impotency is not due to sex muscle deterioration or sperm dysfunction, but rather by defects in turning, vulva-location, and spicule-insertion behaviors (Guo *et al.* 2012; Chatterjee *et al.* 2013). As males age, sex muscle excitability increases (Guo *et al.* 2012). However, transient starvation during late L4 development to early adulthood can reduce adult muscle excitability in the spicule intromission circuitry, and can extend mating potency via UNC-103 and EGL-2 potassium channels (LeBoeuf *et al.* 2011; Guo *et al.* 2012).

SIR-2.1 NAD^+-dependent histone deacetylase is required to maintain mating in aging males (Guo and Garcia 2014). *sir-2.1* mutant males have a hyperexcitable spicule intromission circuit that manifests as a defect in sperm transfer. *sir-2.1* mutant males display altered expression of metabolic genes, enhanced metabolism, and dysregulated expression of antioxidant genes. Hence, SIR-2.1 may protect males from oxidative damage and the effects of aging. These observations are consistent with studies demonstrating that males metabolize sugars and fats differently than hermaphrodites (Tan *et al.* 2011; Liggett *et al.* 2015).

Conclusions

As in many organisms, biological sex has deep influences on the development, structure, and function of the *C. elegans* nervous system. The study of differences between the sexes has provided insights into many biological processes, including neural precursor development, cell fate specification, and differentiation; programmed cell death; synaptogenesis and the formation of circuits; the development and functions of sensory cilia; sensory signaling pathways; the genetic and neural specification of innate behaviors; state-dependent behavioral plasticity; internal and external control of stress responses and life span; signaling mechanisms between the germline and the nervous system; social communication and the roles of EVs; and sexual selection and evolution. These studies have also a profound impact on our understanding of a variety of human disorders, including ciliopathies (Reiter and Leroux 2017), and are likely to shed light on the many neurological and neuropsychiatric conditions that exhibit sex biases in incidence or severity (Loke *et al.* 2015). With the completion of the male connectome, future work is likely to be just as fruitful, and we expect the coming years to see important advances in understanding the neural control of behavior; the mechanisms by which circuits transform sensory input into behavioral transitions; the role of biological sex as a dimension of internal state; the molecular genetic mechanisms that couple sex determination to specific sexually differentiated features of the nervous system; the role of sex differences in the structure of shared circuitry in the *C. elegans* brain; the evolutionary bases for sex-specific features of the nervous system; and the mechanisms and functions of EV release by cilia.

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