One template, two outcomes

One template, two outcomes: How does the sex-shared nervous system generate sex-specific behaviors?

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Abstract

Sex-specific behaviors are common in nature and are crucial for reproductive fitness and species survival. A key question in the field of sex/gender neurobiology is whether and to what degree the sex-shared nervous system differs between the sexes in the anatomy, connectivity and molecular identity of its components. An equally intriguing issue is how does the same sex-shared neuronal template diverge to mediate distinct behavioral outputs in females and males. This chapter aims to present the most up-to-date understanding of how this task is achieved in *C. elegans*. The vast majority of neurons in *C. elegans* are shared among the two sexes in terms of their lineage history, anatomical position and neuronal identity. Yet a substantial amount of evidence points to the hermaphrodite-male counterparts of some neurons expressing different genes and

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forming different synaptic connections. This, in turn, enables the same cells and circuits to transmit discrete signals in the two sexes and ultimately execute different functions. We review the various sex-shared behavioral paradigms that have been shown to be sexually dimorphic in recent years, discuss the mechanisms that underlie these examples, refer to the developmental regulation of neuronal dimorphism and suggest evolutionary concepts that emerge from the data.

1. Introduction

Biological sex impacts brain function at every level, from individual neuronal function to animal behavior. Males and females respond to environmental or intrinsic sensory cues and transform the input into sexually-dimorphic behaviors. Examples range from male-associated aggression and courtship rituals to female-associated nesting and maternal care. In both vertebrates and the nematode *Caenorhabditis elegans* there are two types of sexual dimorphisms in the brain: (1) neurons (or groups of neurons) that exist only in one of the sexes; (2) neurons that are shared by both sexes but are functionally connected and interrelated to one another in a sex-specific manner (Li & Dulac, 2018; Sulston & Horvitz, 1977; White, Southgate, Thomson, & Brenner, 1986; Yang & Shah, 2014). While sex-specific neurons induce or modulate many dimorphic behaviors, the information they acquire and process must be integrated into the sex-shared nervous system. The role of sex-specific neurons has been studied extensively in various organisms (García, 2014; Kimura, Ote, Tazawa, & Yamamoto, 2005; Mason, Rabinowitz, & Portman, 2008; Ruta et al., 2010; Ryan et al., 2014; Sammut et al., 2015; Srinivasan et al., 2008), but we have only recently begun to understand how synaptic connectivity changes between sex-shared neurons generate dimorphic behaviors (Bayer & Hobert, 2018; Fagan et al., 2018; Hilbert & Kim, 2018; Kohl, Ostrovsky, Frechter, & Jefferis, 2013; Oren-Suissa, Bayer, & Hobert, 2016; Weinberg, Berkseth, Zarkower, & Hobert, 2018).

Apart from the traditional advantages of *C. elegans*, which have contributed to its success as a model organism (e.g. transparency, genetic amenability, short life cycle and invariant developmental program), two additional unique features converge in *C. elegans* to make it a particularly suitable model for the study of sexual dimorphism in the nervous system. First, it is the only organism for which the full connectome of both sexes is available (Cook et al., 2019; Jarrell et al., 2012; White et al., 1986), an accomplishment we will address at length below. Second, unlike vertebrate model organisms, the sexual identity of *C. elegans* somatic cells, neurons included,

is determined initially by the genetic sex (XX for the hermaphrodite nervous system, X0 for male fate; Fig. 1A), without the involvement of non-autonomous hormonal regulation (with one known exception that will be discussed below; Lawson, Wexler, Wnuk, & Portman, 2020). This allows

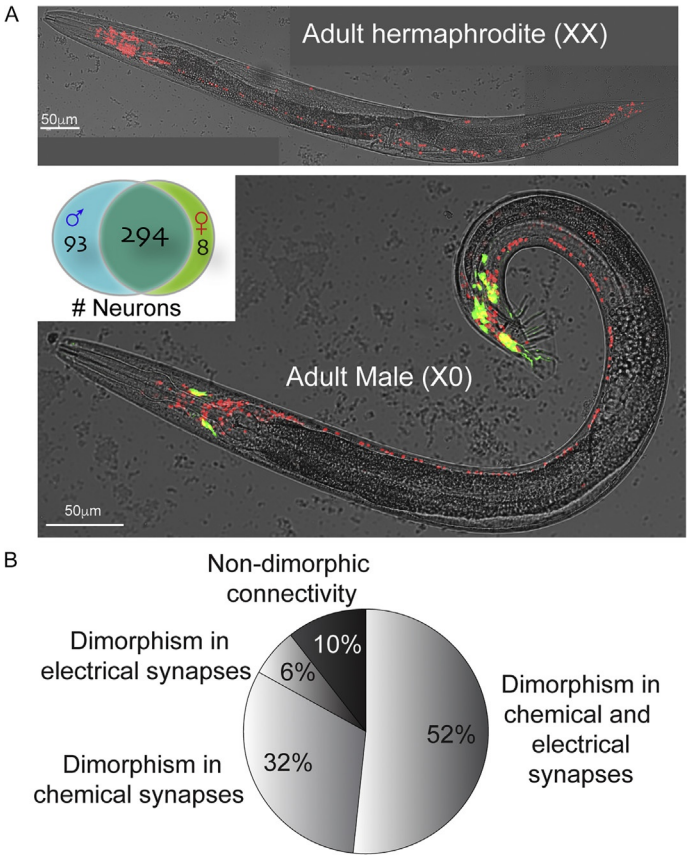

Fig. 1 The nervous system of *C. elegans* in a sexual context. (A) Composite images labeling the nervous system of both sexes. Sex-shared neuronal cell nuclei are labeled in red, male-specific neurons are marked in green. DIC background is added for anatomical orientation. Transgenes are otIs355[rab-3p::NLS::tagRFP]; bxIs14[pkd-2p::GFP]. Inset: the majority of neurons (294 out of 302 for the hermaphrodite) are shared between the sexes, presented as a Venn diagram. (B) Analysis of the published connectome data (Cook et al., 2019) reveals that 90% of the neuronal cell classes are engaged in some form of dimorphic wiring (Dimorphic connectivity was considered as such for all comparisons with a significant *Z*-score. Left/right/dorsal/ventral pairs have been grouped for simplicity). Cook et al. used the variance in connectivity between left/right homologs in the hermaphrodite data to judge how much variance would be expected between the hermaphrodite and male datasets if there were no sex differences. The analysis took into account 124 cells for which connectivity data has been documented.

the *C. elegans* investigator to convert the sexual identity of a neuron of choice to the alternative identity (e.g. from male to female) without directly affecting the sexual fate of the rest of the body (Lee & Portman, 2007; Mehra, Gaudet, Heck, Kuwabara, & Spence, 1999; White et al., 2007) and then examine the subsequent effect on behavior. Although poorly explored, an autonomous role for genetic sex in the function of the brain and other somatic tissues in vertebrates has also been documented (Arnold, 2019; McCarthy & Arnold, 2011; Yang et al., 2006).

In this review, we examine how the genetic sex of sex-shared neurons and circuits affects universal behaviors such as locomotion and sensory perception. We do not address the contribution of sex-specific neurons to sex-specific behaviors (e.g., HSN and VC motor neurons for egg laying in hermaphrodites), nor the involvement of sex-shared neurons in sex-specific behaviors (e.g. how the shared defecation circuit is integrated into the sperm transfer circuit in males, see Cook et al., 2019; LeBoeuf & García, 2017). Several comprehensive reviews have been written on these topics in recent years (Barr, García, & Portman, 2018; Garcia & Portman, 2016; Oren-Suissa & Hobert, 2017; Portman, 2017).

Here we will elaborate on questions regarding common behavioral traits, with special attention to recent advances in the field: Do males and females move differently? Do they differ in their response to the same environmental cues? Do they integrate sensory information differently? Do they execute fundamental neural paradigms (learning, memory, decision making) in an identical manner? If not, what is the relative contribution of sex-shared neurons to dimorphic behaviors, and what are the underlying mechanisms that diverge between the sexes? Can the answers to these questions expand our knowledge about the sex-biased distribution of neuropsychiatric disorders and neurodegenerative diseases?

We begin by briefly describing the principles of sex determination in *C. elegans*. Next, we present those shared behavioral paradigms for which sexual dimorphism has been documented. We then delve into the mechanisms that drive such behavioral differences between the sexes, first addressing differences in the anatomical wiring of neurons and then moving to molecular differences in gene and protein expression. In the final section, we discuss the developmental aspects of neuronal dimorphism.

2. Sex determination in *C. elegans*

Sex differences in vertebrate brains are the result of two factors: A cell-non-autonomous influence by gonadal hormones, which determines sex

differences (McCarthy, 2010; McEwen & Milner, 2017), and an understudied cell-autonomous influence of the sex chromosome complement of a cell (e.g. the male-specific Sry gene, located on the Y chromosome, is expressed in specific regions of the adult brain and is required for specific aspects of brain function, whereas genes on the X chromosome may be expressed at different levels in both sexes) (Jazin & Cahill, 2010; McCarthy & Arnold, 2011; McCarthy, Nugent, & Lenz, 2017). Sex chromosome-dependent/hormone-independent determinants of sexual identity are also demonstrated by the observation that some sexually-dimorphic gene expression patterns in the mouse brain precede gonadal differentiation (Büdefeld, Grgurevic, Tobet, & Majdic, 2008; Dewing, Shi, Horvath, & Vilain, 2003). Thus, sex chromosomes can exert both specific and broad influences on the developing brain.

As in many other species, the basic sexual identity of an individual *C. elegans* animal is dictated by its sex chromosome complement. Animals carrying two copies of the X chromosome will develop as hermaphrodites, whereas those carrying one X chromosome (designated X0) will become males. Hermaphrodites are so called because their gonad produces self-sperm for a limited time window early in life, before switching to oocyte production for the rest of their lives. Hence, hermaphrodites can self-fertilize to produce progeny, while males need to find a hermaphrodite mate in order to copulate. Outside their gonad, hermaphrodites are somatic females, having female genitalia and laying progeny.

The molecular events that translate sex chromosome count into a sexual identity in *C. elegans* have been elucidated quite extensively and reviewed in depth (Barr et al., 2018; Wolff & Zarkower, 2008). Briefly, the ratio of sex-chromosome-to-autosome numbers (being higher in hermaphrodites) determines the balance between opposing cues that converge on the expression of the transcription factor XOL-1. XOL-1 activates a well-described genetic pathway that culminates in the inhibition of the master sex-determining transcription factor TRA-1A. In hermaphrodites, XOL-1 activity is off and TRA-1A is on, while the opposite occurs in males (Hunter & Wood, 1990; Rhind, Miller, Kopczynski, & Meyer, 1995). TRA-1A is mostly a transcriptional repressor, driving hermaphrodite fate by actively inhibiting male fate target genes (Berkseth, Ikegami, Arur, Lieb, & Zarkower, 2013; Hunter & Wood, 1990; LeBoeuf & García, 2017; Yi, Ross, & Zarkower, 2000). As mentioned above, TRA-1A activity determines the sexual fate in every somatic cell autonomously. By experimentally manipulating TRA-1A activity in any given somatic cell specifically, one can sex-reverse the fate of individual cells of choice without

affecting the genetic sex of the rest of the body (Lee & Portman, 2007; Mehra et al., 1999; White et al., 2007). These cell-specific manipulations have confirmed the instructive and autonomous role of TRA-1 in the sexual differentiation of the nervous system, and have proven extremely useful in teasing out the key neurons that instruct sex specificity in sexually-dimorphic circuits (Lee & Portman, 2007; Lum, Kuwabara, Zarkower, & Spence, 2000; Mehra et al., 1999; Mowrey, Bennett, & Portman, 2014; Oren-Suissa et al., 2016; White et al., 2007).

Several TRA-1 targets have been identified and are summarized in Table 1. These include *dmd-3* and *mab-3* (Berkseth et al., 2013; Mason et al., 2008; Yi et al., 2000), which encode for transcription factors of the conserved DMD (Doublesex/MAB-3 domain) family and regulate sexual differentiation in diverse organisms (Kopp, 2012; Zarkower, 2001); *fog-3*, regulator of sperm differentiation whose repression by TRA-1A promotes oogenesis (Chen & Ellis, 2000; Jin, Kimble, & Ellis, 2001); *ceh-30* and *egl-1*,

Table 1 Reported direct targets of TRA-1 transcription factor.

Gene name	Product/function	References
egl-1	BH3-only protein, activates cell death	Conradt and Horvitz (1999)
fog-3	Transcription factor, controls sperm fate	Chen and Ellis (2000)
ceh-30	Homeobox transcription factor, anti-apoptotic	Peden, Kimberly, Gengyo-Ando, Mitani, and Xue (2007)
mab-3dmd-3	Transcription factors of the DMD (Doublesex/MAB-3 domain) family, regulate sex differentiation	Yi et al. (2000) and Mason et al. (2008)
tra-1, xol-1	Transcriptional repressors in the sex determination and dosage compensation pathways	Hargitai et al. (2009) and Berkseth et al. (2013)[a]
lin-4, lin-42, lin-28, lin-29	Heterochronic pathway	Berkseth et al. (2013)[a] and Pereira et al. (2019)[b]
unc-6	Netrin guidance cue	Weinberg et al. (2018)
goa-1	G protein subunit Gα(i/o)	Kutnyánszky et al. (2020)

[a]Berkseth et al. conducted a ChIP-seq on TRA-1 and report many potential targets, most of which have not been validated experimentally.
[b]*lin-29* contains *tra-1* binding sites which have not been validated experimentally.

which direct sex-specific apoptotic removal of individual neurons (Conradt & Horvitz, 1999; Peden et al., 2007); *goa-1*, a G protein subunit that controls male-associated behaviors (Kutnyánszky et al., 2020); and *unc-6*, the *C. elegans* netrin ortholog, whose expression is downregulated by TRA-1 in specific cells in hermaphrodites during development (Weinberg et al., 2018). TRA-1A has also been suggested to directly control the timing of sexual maturation by binding to the loci of developmental clock genes (heterochronic pathway, see below; Berkseth et al., 2013). Thus, TRA-1A regulates sexual differentiation by directly repressing male fate genes at multiple levels and tissues during development.

Nonetheless, the long-standing view that TRA-1A expression is strictly limited to hermaphrodites in the adult soma has been challenged very recently by two studies that demonstrate enduring TRA-1A expression in small subsets of the adult male nervous system (Bayer, Sun, Rafi, & Hobert, 2020; Lawson et al., 2020). Why does TRA-1A remain expressed in specific male neurons is still a mystery, but it seems to allow the male to retain some behavioral plasticity in response to a changing environment (Lawson et al., 2020).

3. Sexual dimorphism in sex-shared behavioral states and traits

Some behaviors are highly sex-specific, such as male courtship or maternal nursing. But other behaviors can be considered universal regardless of chromosomal sex, like locomotion, awareness and response to perils, learning and memory. At face value, these behaviors are shared and thus need not necessitate a dimorphic aspect. Yet, several shared behavioral traits have been shown to exhibit sex differences in *C. elegans*, and are briefly presented below.

3.1 Locomotion

Males and hermaphrodites display inherent differences in the mechanical properties of locomotion under naïve conditions (i.e., in the absence of environmental stimulation), such as higher velocity and body bend frequency in males (Mowrey et al., 2014; Pereira et al., 2019; Suo et al., 2019). These differences have been attributed to dimorphism in both the musculature and the nervous system (Mowrey et al., 2014). Interestingly, the shared core locomotion circuit, including command interneurons and the cholinergic and GABAergic motor neurons, do not seem to contribute

to this dimorphism. Instead, the sensory nervous system determines the dimorphic locomotion patterns, although the precise sensory neurons that drive these differences in the two sexes have yet to be identified (Mowrey et al., 2014).

In the presence of food, *C. elegans* hermaphrodites spend most of their time feeding on the bacterial lawn while moving slowly, a behavioral state termed "dwelling," shifting occasionally to a faster exploratory state named "roaming" (Flavell et al., 2013; Suo et al., 2019). Males, on the other hand, will leave the food more readily (when no hermaphrodites are present there) and engage in mate-searching behavior (Lipton, Kleemann, Ghosh, Lints, & Emmons, 2004; Suo et al., 2019). The two sexes differ greatly in the proportion of time allocated to each behavioral state (Suo et al., 2019). These behaviors are generated by sex-shared core circuits (Barrios, Ghosh, Fang, Emmons, & Barr, 2012; Flavell et al., 2013), as discussed in Section 5.

3.2 Chemo-avoidance

C. elegans senses and avoids hazardous signals in the environment through a sensory circuit known as the "chemo-avoidance circuit" (Hilliard, Bargmann, & Bazzicalupo, 2002). Although the core circuit contains only sex-shared neurons, in some conditions males and hermaphrodites can respond differently to the same aversive stimulus (Oren-Suissa et al., 2016; M.O.-S. and Y.S. personal observations). The underlying mechanism involves dimorphic wiring patterns, as elaborated in Section 4.

3.3 Chemo-attraction

The two sexes display discrete sensory responses to environmental odorants and cues that signal the presence of food or a potential mate. For example, food odorants, such as diacetyl, attract *C. elegans* hermaphrodites with much higher potency than males (Lee & Portman, 2007; Ryan et al., 2014). On the other hand, wild-type *C. elegans* males, but not hermaphrodites, are attracted to low concentrations of the sex pheromone (mating attractant) ascaroside C9 (or #3), through the combined action of the sex-shared ASK and male-specific CEM neurons (Macosko et al., 2009; Srinivasan et al., 2008). The differences at the molecular and circuit levels that account for these dimorphic behaviors are described below. A different set of studies using hermaphrodite-conditioned medium instead of pure ascarosides found male-specific attraction to involve the shared olfactory neurons AWA and AWC together with CEM and ASK (White et al., 2007; White & Jorgensen, 2012).

3.4 Associative learning

Worms can learn to associate two signals and, following a training period, adjust their behavior toward one signal by the mere presence of the other. Sakai et al. demonstrated that males and hermaphrodites can exhibit different associative abilities (Sakai et al., 2013). When trained to associate the absence of food with salt, both hermaphrodites and males migrated efficiently away from a salt source. However, when hermaphrodites were present on the male training plate, males lost their salt-aversion behavior. This suggests that for males, association of salt with a potential mate overrides the baseline association of salt with starvation (Sakai et al., 2013). The underlying neuronal and molecular basis for this dimorphism has not yet been elucidated.

As mentioned above, we exclude here behaviors that are overtly sex-specific, such as egg laying in hermaphrodites or copulation in males, behaviors that cannot occur in the opposite sex due to lack of the relevant organs and cells, and are reviewed elsewhere (Barr et al., 2018; Emmons, 2018). Nevertheless, the neural circuits that mediate such behaviors usually interact with sex-shared neurons and thus may employ similar mechanisms to those discussed below to diversify the shared nervous system in the two sexes.

4. Mechanisms that generate a dimorphic sex-shared nervous system: Dimorphic connectivity

One potential way to obtain disparate behavioral outcomes from a single neuronal blueprint is by the differential wiring of its components. Indeed, a comparative look at the connectome of the two sexes reveals a profusion of dimorphic circuits (Cook et al., 2019; Emmons, 2018). Cook et al. have recently published new wiring diagrams (connectomes) of the nervous system of both sexes of *C. elegans*, representing a major step toward understanding how brain structure and function correlate. This study reconstructed the male anterior region and included new connections to end organs, sub-lateral motor neurons, and a number of gap junctions. It also increased the resolution of the connectomes by including the connectivity of individual neurons within each neuron class. Overall, the nervous system is determined to be a quasi-layered, largely feedforward architecture, with sensory information passing through three layers of interneurons before reaching motor neurons, which ultimately confer function. On top of this feedforward information flow, different layers of neurons also exhibit extensive crosstalk. Remarkably, Cook et al. found that around 30% of connections differ in synaptic strength between the sexes. The authors compared

the connectivity between cell classes, analyzing connectivity data between individual pairs, and computed a *z*-score for each connection to judge the difference in connection strengths between the sexes, normalized by its estimated variance. Surprisingly, 102 out of the 114 pairs of sex-shared neurons for which a connection was reconstructed, were found to be engaged in some form of dimorphic connectivity (either a different number or strength of chemical or electrical synapses between the sexes) (Fig. 1B). This number, however, may be an overestimate, since it is unclear how much of the variance between the sexes is attributed to inter-animal variability, and only a handful of the predicted dimorphic connections have been validated in vivo by trans-synaptic labeling methods (Cook et al., 2019; Oren-Suissa et al., 2016).

One case of sex-specific wiring patterns that has been studied in depth is the circuit for chemo-avoidance (Oren-Suissa et al., 2016). The core circuit is composed mainly of four sex-shared neurons that connect in a highly dimorphic manner (Fig. 2A). For example, the sensory neuron PHB makes mutually exclusive connections to AVA and AVG interneurons in adult hermaphrodites and males, respectively. It has been shown that in juvenile worms both connections (PHB > AVA and PHB > AVG) form, and it is only during sexual maturation that one connection is eliminated in a sex-specific manner (Oren-Suissa et al., 2016). These dimorphic synaptic pruning events depend on the sex-specific activity of several transcription factors (TFs), although the precise interplay between them has not been fully elucidated. First, the master regulator of sex determination, TRA-1, was found to control the hermaphrodite-specific removal of the PHB > AVG connection (Weinberg et al., 2018). In males, a different set of TFs, belonging to the DMD (Doublesex/MAB-3 domain) family of conserved TFs is active in AVG (Oren-Suissa et al., 2016). Mechanistically, the maintenance of the PHB > AVG synapse in males has been linked to the male-specific release of UNC-6/Netrin from the post-synaptic AVG cell. UNC-6 in this context acts as a paracrine cue bound by the netrin receptor UNC-40 on the neighboring PHB to stabilize the synapse in males (Fig. 2B) (Weinberg et al., 2018). In hermaphrodites, TRA-1 directly binds and represses *unc-6* transcription, thereby preventing synapse stabilization in a mechanism involving the regulated degradation of UNC-40 in hermaphrodite PHB (Fig. 2B) (Salzberg et al., 2020). UNC-40 carries a conserved degradation motif named cdc4 phosphodegron (CPD) in its cytoplasmic tail, that can be bound by an SCF (for **S**KP, **C**ullin and **F**-box protein) complex containing the E3 ubiquitin ligase SEL-10/FBW7. In hermaphrodite PHB

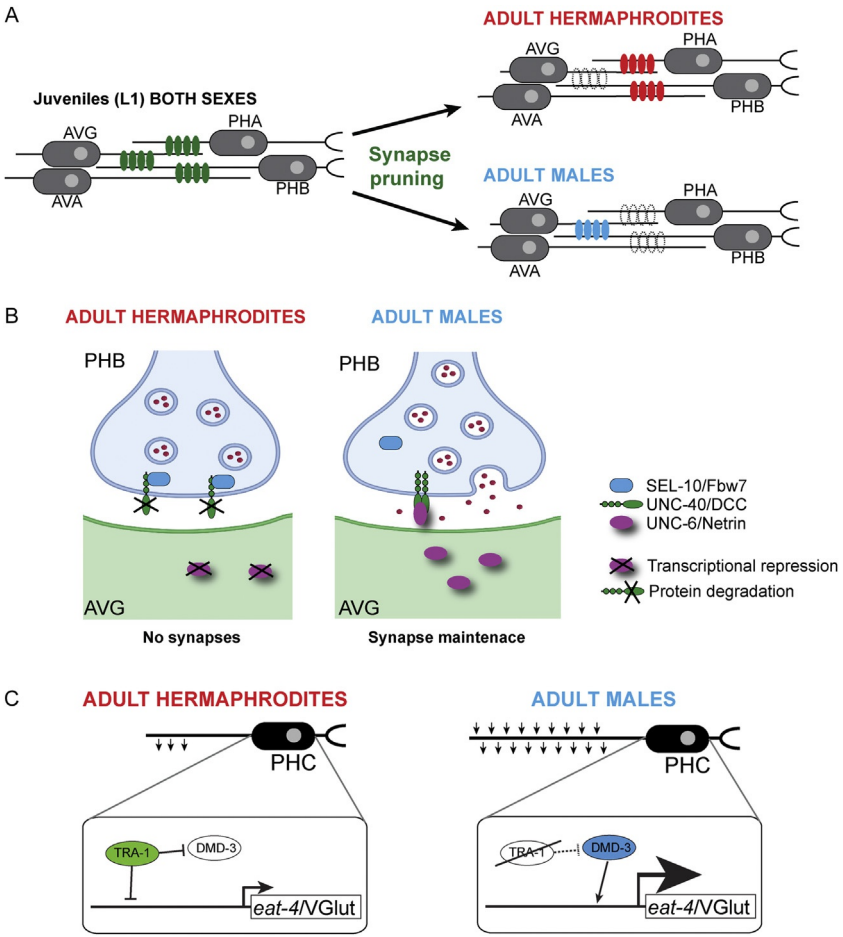

Fig. 2 Sexually-dimorphic connectivity patterns. (A) The phasmid neurons in the tail form identical connections in both sexes in juvenile animals, which later become dimorphic due to selective pruning of different synapses in each sex (dotted circles) (Oren-Suissa et al., 2016). (B) Sex-specific removal of synapses involves the targeted proteasomal degradation of the netrin receptor UNC-40 only in hermaphrodites. In adult hermaphrodites no UNC-6 is secreted from AVG (Weinberg et al., 2018), and the E3 ligase SEL-10 is able to bind the UNC-40 netrin receptor via binding to a conserved CPD motif and target it for degradation (Salzberg et al., 2020). UNC-40 degradation leads to synapse removal only in hermaphrodites, not males. In males, netrin is secreted from AVG and protects UNC-40 from SEL-10 binding in an unknown manner. (C) PHC becomes a hub neuron in males in part due to the upregulation of the glutamate transporter *eat-4* by the TF *dmd-3*. In hermaphrodites, *eat-4* expression remains low by TRA-1 repression (Serrano-Saiz, Oren-Suissa, Bayer, & Hobert, 2017).

UNC-40 is degraded through SEL-10 activity, while in male PHB, the secretion of UNC-6 from AVG protects UNC-40 from SEL-10-mediated degradation, which then leads to synapse maintenance. These results highlight an emerging role for regulated protein degradation as a means to shape dimorphic circuits via synapse elimination in a spatial, temporal and sex-dependent manner (Salzberg et al., 2020).

Early life history events, such as starvation, can have long-term effects on dimorphic circuits (Bayer & Hobert, 2018; Ryan et al., 2014). Males that have undergone a starving period during larval stages will retain hermaphrodite-like connectivity and consequent behavioral properties in the chemoavoidance circuit (i.e., they remain sensitive to hazardous chemicals at the expense of their mating efficiency; Bayer & Hobert, 2018). The information about the nutritional state of the animal is relayed to the circuit through monoamine signaling. Serotonin secretion from ADF has been shown to mediate the satiety signal in males, while octopamine secreted from RIC suppresses this signal under starvation conditions (Bayer & Hobert, 2018). Although serotonin biosynthesis has been shown to be higher in male ADF, how this is regulated dimorphically has not been addressed yet.

Another phasmid neuron that displays highly dimorphic connectivity is PHC. In hermaphrodites, this neuron functions strictly as a sensory neuron, while in males it receives heavy input from both male-specific and shared neurons. By silencing PHC, a covert behavioral dimorphism is revealed: hermaphrodites with no PHC activity partially lose the escape response to a tail harsh-touch while males do not (Serrano-Saiz et al., 2017); this is likely because this neuron is repurposed to mating behaviors at the expense of its role as a mechanosensor. Similar to the PHB > AVG male-specific connection, male-specific differentiation of PHC into a hub neuron has also been shown to depend on the function of a DMD TF, *dmd-3*, which cell-autonomously induces synaptic and anatomical changes in male PHC to accommodate for its enhanced sex-specific roles (Fig. 2C) (Serrano-Saiz et al., 2017).

Dimorphism in circuit connectivity can manifest also in the relative weight that identical synapses are endowed with, in the two sexes. High concentrations of the ascaroside C9, which likely signals a crowded environment, strongly repels wild-type hermaphrodites while only mildly deterring males (Jang et al., 2012; Srinivasan et al., 2008). These dimorphic responses stem from sex-specific crosstalk between sex-shared neurons. The core circuit that senses and responds to C9 has been suggested to be composed of seven sex-shared neurons (Jang et al., 2012). C9 can potentially activate the amphid sensory neuron ADL in both hermaphrodites and males to elicit

repulsion. In males, however, an antagonistic input from ASK counteracts the activation of ADL to suppress the avoidance response (reversal) (Jang et al., 2012). The response of both sexes to C9 changes dramatically under conditions of low neuropeptide signaling through the neuropeptide Y receptor *npr-1*- hermaphrodites become indifferent to high C9 concentrations whereas males are attracted to it. The reason is that low *npr-1* activity tips the balance of the circuit from ADL-mediated repulsion to ASK-mediated attraction. This occurs through *npr-1's* effect on the hub neuron RMG, which forms electrical synapses with both ADL and ASK and can, thus, modulate their function (Fig. 3). Hence, neuropeptide signaling can

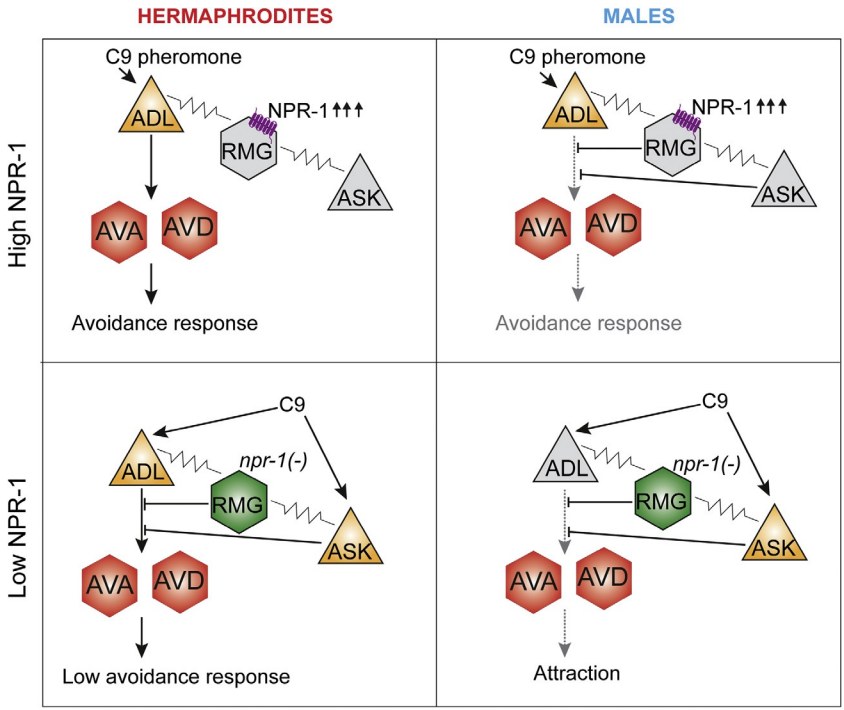

Fig. 3 Sexual identity and neuropeptide signaling co-modulate the response to the ascaroside C9. Under high NPR-1 activity (top panels), hermaphrodites avoid C9 through ADL-mediated avoidance response. Males (right upper panel) are less sensitive to C9 because ASK activity diminishes the signal from ADL in the RMG circuit. In addition, the recent head connectivity data for the male (Cook et al., 2019) reports the absence of ADL to AVA synapses, which might also account for the behavioral differences. It is uncertain that ASK and RMG form electrical synapses in males (wormwiring.org). When NPR-1 levels are low (bottom), males become attracted to C9, because stronger ASK-RMG activity overrides the signal from ADL in the circuit. ASK activity in hermaphrodites neutralizes the ADL avoidance response. *Modified with permission from (Jang et al., 2012).*

modulate dimorphic circuits by fine tuning the relative weight of specific subsets of neurons and synapses within the circuit. New male connectivity data (Cook et al., 2019) suggests that there are some dimorphisms in the circuit that mediate the pheromone sensation (Fig. 3). Further investigation is required to validate these dimorphic connections and evaluate the behavioral responses in light of the connectivity data. Another intriguing research direction is the analysis of connectivity in wild isolates.

5. Molecular mechanisms that generate a dimorphic sex-shared nervous system: Dimorphic gene expression/molecular profile

In some cases, the connectivity pattern of a particular neuron or circuit seems superficially identical in hermaphrodites and males, yet the behavioral readout of these neurons' activity is sexually dimorphic as a result of differential gene expression. For example, sex-limited expression of odorant GPCR receptors partially underlies the opposite attraction of the two sexes to food and pheromones (Ryan et al., 2014; Wan et al., 2019). Hermaphrodites are attracted to food at least in part through high expression levels of the diacetyl receptor ODR-10 in the olfactory neuron AWA (Fig. 4). Males normally display much weaker attraction to food due to little

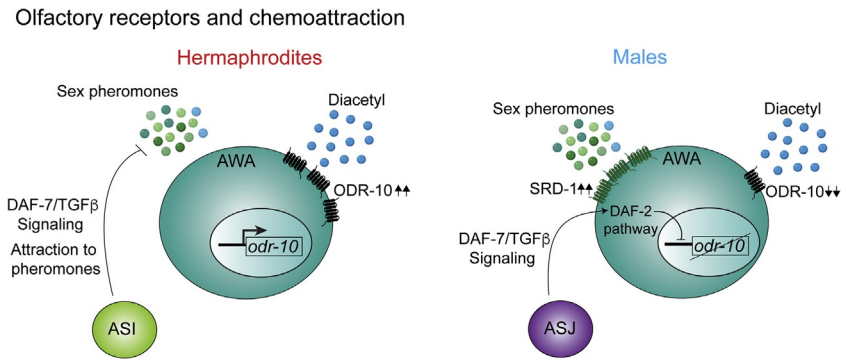

Fig. 4 Dimorphic receptor expression in AWA. Left: Hermaphrodites are attracted to diacetyl due to high expression levels of ODR-10 in AWA. Attraction to sex pheromones is suppressed in hermaphrodites through DAF-7 signaling, likely secreted from ASI (White & Jorgensen, 2012). Right: Fed males are attracted to sex pheromones through expression of the pheromone receptor SRD-1 in AWA (Wan et al., 2019). ODR-10 expression in fed males is low due to the inhibitory action of DAF-7 secreted from ASJ, that activates DAF-2 signaling in AWA, ultimately leading to downregulation of *odr-10* expression (Wexler, Miller, & Portman, 2020).

expression of ODR-10 in AWA (Lee & Portman, 2007; Ryan et al., 2014), but express high levels of the GPCR SRD-1, which mediates attraction to volatile pheromones (Wan et al., 2019) (Fig. 4). Therefore, males are attracted to a drop of hermaphrodite-conditioned medium while hermaphrodites remain indifferent. Importantly, forced expression of either receptor in AWA of the opposite sex switches its olfactory preferences, demonstrating that dimorphic behavioral preferences in this case can be determined purely by the membrane composition of the sensory neuron, irrespective of possible dimorphic components that may lie downstream in the circuit (Ryan et al., 2014; Wan et al., 2019).

Many experimental setups have been employed for pheromone attraction assays, leading to the discovery of multiple circuits and mechanisms (McGrath & Ruvinsky, 2019), with sometimes seemingly contradictory findings. For example, the role of the SRD-1 receptor (Wan et al., 2019) or the CEM, ADL and ASK neurons (Srinivasan et al., 2008; White et al., 2007; White & Jorgensen, 2012) in the response to pheromones has not been confirmed by a different study that used a purified ascaroside mixture as the attractant (Fagan et al., 2018). This report identified the sex-shared ADF sensory neuron as critical for promoting pheromone attraction in males by overriding an otherwise innate repulsive reaction seen in hermaphrodites (Fagan et al., 2018). The authors suggest that the innate repulsion in both sexes is mediated by the ADL neurons (Jang et al., 2012). No dimorphic receptor expression was reported that could account for the male-specific response of ADF to ascarosides.

The low expression of the ODR-10 receptor in fed males has been linked to the dimorphic action of two pathways: DAF-7/TGFβ and DAF-2/insulin/IGF-1-like signaling (IIS) (Wexler, Miller, & Portman, 2020). Under normal conditions, the DAF-7 cue is released from the ASJ neuron in males only (Hilbert & Kim, 2017; Wexler et al., 2020). DAF-7 activates the secretion of an insulin ligand that ultimately activates the *daf-2* IIS pathway cell autonomously in the AWA cell to repress *odr-10* expression (Wexler et al., 2020) (Fig. 4). Upon starvation, males will transiently upregulate *odr-10* expression to prioritize eating over mate searching (Ryan et al., 2014). *odr-10* upregulation in starved males requires TRA-1A activity as a permissive cue in an unidentified neuron other than AWA itself (Lawson et al., 2020). Therefore, it seems that hormonal pathways such as IIS can modulate the sexual state of adult male neurons in response to nutritional cues, rendering the sexual state of neurons more plastic than previously appreciated.

Unexpectedly, starved hermaphrodites seem to use different mechanisms than starved males to downregulate *odr-10* expression after being re-fed. Elegant experiments by (Wexler et al., 2020) revealed that males suppress *odr-10* in response to chemical cues emanating from the food source without the need to actually consume it, while hermaphrodites respond to the physiological change in metabolic state upon food consumption. This difference may be explained by the evolutionary need of hermaphrodites to be more attuned to their internal physiological state, since it determines the survival of the offspring they carry.

Interestingly, while wild-type hermaphrodites do not exhibit attraction to a hermaphrodite-conditioned medium, *daf-7* mutant hermaphrodites become attracted like males (White & Jorgensen, 2012). This reversed behavior requires an ensemble of sex-shared sensory neurons (AWA, AWC, ASK) and interneurons (AIA, AIB, AIY and AIZ), suggesting that DAF-7 normally represses male-like attraction to pheromones in hermaphrodites. Therefore, the same signaling cue elicits opposite behaviors in the two sexes. The underlying mechanism is unknown, but may involve the dimorphic neuronal source for DAF-7 (ASI in hermaphrodites, ASJ in males; Hilbert & Kim, 2017) (Fig. 4). Sex-reversal experiments on these two neurons in males will be necessary to address this issue.

In the absence of a pheromone signal from mates, males will advertently leave a food source in favor of an exploratory behavior that reflects their reproductive motivation. This food-leaving behavior depends on the action of the PDF-1 signaling pathway in a set of sex-shared neurons (AIM, PQR, PHA, URY; Barrios et al., 2012). However, Barrios et al. found that PDF-1 and its receptor PDFR-1 have similar expression patterns in the two sexes. Thus, the dimorphic behavioral output is likely due to downstream differences in the molecular properties and/or connectivity of the involved neurons. Indeed, at least two of the neurons in this circuit, AIM and URY, make abundant connections in hermaphrodites but very few in males (Cook et al., 2019; wormwiring.org). Intriguingly, PDF-1 exerts roaming behavior in hermaphrodites, which may be considered analogous to the mate searching, exploratory behavior in males, but it was shown to involve a completely different circuit, one that includes the sex-shared neurons PVP, AVB, RIA, AIY and RIM (Flavell et al., 2013). This offers a unique example of a similar behavior in the two sexes (roaming in hermaphrodites, mate searching in males) that is induced by the same signaling pathway but that involves highly dimorphic circuits. It would be interesting to address what evolutionary forces drove the separation into distinct circuits in the two sexes to mediate a similar behavior.

The relative time the two sexes spend in the dwelling and roaming states is, too, dimorphic (Suo et al., 2019). Dopamine plays a key role in this difference, since in *cat-2* mutants, in which dopamine biosynthesis is blocked, males roam less and hermaphrodites roam more to reach similar ratios. The effects of dopamine in the two sexes are highly dimorphic in several respects. First, different dopamine receptor combinations mediate its effect in the two sexes. Second, in hermaphrodites, dopamine acts through inhibition of octopamine neurotransmission, whereas in males there is no apparent contribution for octopamine. Third, octopamine activates the sex-shared SIA neuron non-autonomously in hermaphrodites but not in males (Suo et al., 2019). How do the dimorphic effects of dopamine and PDF-1 on food leaving interact with each other is a matter for future investigation.

Another mechanism that generates sexual dimorphism involves sex-specific modifications in neurotransmission. Pereira et al. have revealed that in juvenile animals of both sexes the interneuron AIM is strictly glutamatergic, based on the expression of the vesicular glutamate transporter EAT-4 (VGlut) (Pereira et al., 2015). However, as males undergo sexual maturation, AIM loses EAT-4 expression and instead upregulates the ACh transporters UNC-17 and CHO-1, while hermaphrodite AIM remains glutamatergic. AIM sex-reversal experiments have shown that these effects are cell autonomous (Pereira et al., 2015). The physiological purpose of this sex-dependent switch is unknown.

6. Developmental aspects of neuronal dimorphism

Genetic sex (sex chromosome complement) endows every cell of the embryo with sexual identity right from the zygote and throughout development, while overt sexualization of the animal (i.e., the appearance of sex-specific cells and organs) occurs mostly during sexual maturation, at the last larval stage. Do sex-shared neurons, despite their superficial identity among the two sexes, acquire sex-distinct properties early in development or do they become sexualized during sexual maturation in unison with the rest of the body? Many recent findings shed light on this topic.

At all the levels examined—behavior, connectivity and molecular profile—the majority of reports point to sexual maturation at the L4 stage as the time point when sexual features emerge in the nervous system. Behaviorally, sex differences in the chemo-avoidance circuit, roaming-to-dwelling ratios or locomotion patterns are absent at early larval stages (Oren-Suissa et al., 2016; Suo et al., 2019). In terms of circuit connectivity, most dimorphic connections arise only during sexual maturation through

the sex-specific pruning of synapses, although few sex-specific synapses have been observed to exist already at juvenile stages (Cook et al., 2019; Oren-Suissa et al., 2016). Furthermore, the lack of widespread sex-specific connectivity in juvenile stages is supported by the finding that TRA-1A activity is largely off in neurons at L1–L2 stages (Bayer et al., 2020; Weinberg et al., 2018). Molecularly, *odr-10* expression in AWA becomes dimorphic only after sexual maturation (Ryan et al., 2014). The same is reported for UNC-17 and CHO-1 in AIM (Pereira et al., 2015) and for UNC-6 expression in the interneuron AVG (Weinberg et al., 2018), to list just a few examples.

What cue then switches on sexual properties in shared neurons as the animal reaches sexual maturity? Two recent works have shown that the timing of sexualization of the shared nervous system is coupled to the developmental clock of other somatic tissues through the activity of a conserved pathway called the heterochronic pathway (Lawson et al., 2019; Pereira et al., 2019), first identified in *C. elegans* (Ambros & Horvitz, 1984). This pathway orchestrates the timely progression of larval development so that growth and differentiation of all somatic tissues occurs in a coordinated manner and in tandem with gonadal maturation (Moss, 2007; Pasquinelli & Ruvkun, 2002). Several important concepts arise from the reports: (1) Like sexual identity, timing of neuronal differentiation is a cell-autonomous decision in *C elegans*. Thus, restoring the expression of the heterochrony regulator *lep-2*/Makorin specifically in the neuron AIM of *lep-2* mutants rescues the timely appearance of its male-characteristic neurotransmitter switch, but not the male-specific loss of *odr-10* expression in AWA, and vice versa (Lawson et al., 2019). (2) Terminal cellular identity in the nervous system is determined by the intersection of multiple transcriptional inputs, such as sex (sex determination pathway), developmental time (heterochronic pathway), and neuronal lineage (neuronal terminal selectors) (Pereira et al., 2019). Therefore, perturbing any of these pathways will disturb the full genetic program of individual neurons, with behavioral consequences. (3) To some extent, the distinction between sex-specific and sex-shared neurons is manifested molecularly, by the restricted expression of the heterochronic regulator *lin-29a* to some sex-shared neurons of the male, but it is never expressed in male-specific neurons (Pereira et al., 2019). This finding suggests that some crosstalk must exist between the sex determination and heterochronic pathways. For example, heterochronic loci such as *lin-29a* and *lin-41* may be under direct transcriptional control of TRA-1A to ensure their male-specific expression (Berkseth et al., 2013).

Whether sex-shared neurons have a common transcriptional fingerprint is an intriguing question that warrants further investigation.

The coupling of somatic developmental programs with acquisition of cellular sexual fate may be coordinated by hormonal signals such as the dafachronic acid nuclear hormone receptor DAF-12, that regulates both heterochronic pathway genes (Bethke, Fielenbach, Wang, Mangelsdorf, & Antebi, 2009; Hochbaum et al., 2011) and TRA-1A expression (Bayer et al., 2020).

7. Future directions and concluding remarks

Darwin described a set of dynamics operating within species that are the evolutionary drivers of sex differences (Darwin, 1859, 1871). Science now views sexual selection and natural selection as distinct, occasionally even opposing, evolutionary forces, highlighted by cases where sexual selection selects for phenotypes that are not favored by natural selection (Zahavi, 1975, 1977). Since sexual selection is the main driving force for the development of sex-specific traits, we hypothesize that the nervous systems of the two sexes have evolved to accommodate changes that maximize fitness for reproduction. Moreover, given the diverse landscape of dimorphic behaviors that emanate from the common neuronal blueprint in the two sexes, one may ask whether they arose in evolution by a single unifying event that enabled sex-specific modification of the blueprint or whether they evolved separately to incorporate different sex-specific functions.

At the molecular level, research in recent years on various organisms suggests that brain sexual differentiation depends on regulators of sex determination, which act early during development to set dimorphic gene expression, which in turn controls neural pathways and behavior (Knoedler & Shah, 2018). With the recent completion of the male connectome, we are starting to fully appreciate the scope of differences between the sexes. A full picture will emerge upon the integration of the connectome data with a systematic analysis of the transcriptome of the two sexes and rigorous molecular studies that take advantage of the *C. elegans* nervous system.

There are potential clinical implications for research into the influence of sex on the nervous system. Many of the genes associated with common neurological diseases, such as Huntington's disease, Alzheimer's disease, posttraumatic stress disorder and schizophrenia, display sexual dimorphism in disease development, pathological processes and recovery mechanisms (Flanagan, 2014; Gilks, Abbott, & Morrow, 2014; Ober, Loisel, & Gilad,

2008). Understanding how genetic mechanisms function to modulate dimorphic circuits could prove beneficial in the development of novel sex-specific therapies.

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