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The development of sex differences in the nervous system and behavior of flies, worms, and rodents

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ABSTRACT

Understanding how sex differences in innate animal behaviors arise has long fascinated biologists. As a general rule, the potential for sex differences in behavior is built by the developmental actions of sex-specific hormones or regulatory proteins that direct the sexual differentiation of the nervous system. In the last decade, studies in several animal systems have uncovered neural circuit mechanisms underlying discrete sexually dimorphic behaviors. Moreover, how certain hormones and regulatory proteins implement the sexual differentiation of these neural circuits has been illuminated in tremendous detail. Here, we discuss some of these mechanisms with three case-studies—mate recognition in flies, maturation of mating behavior in worms, and play-fighting behavior in young rodents. These studies illustrate general and unique developmental mechanisms to establish sex differences in neuroanatomy and behavior and highlight future challenges for the field.

1. Introduction

Sex differences in social behaviors are common among sexually reproducing animals and can be impressively dramatic. Some of these differences are dimorphic, in which a behavior, such as a certain mating behavior, is displayed by one or the other sex. However, sex differences can also be quantitative, in which a particular behavior, present in both sexes, differs on average for each sex (McCarthy et al., 2012). Many sex differences in behavior are learned from experience, but a great deal of others—*e.g.*, mating, territoriality, play-fighting, aggression, and parenting—are largely innate and can be displayed by animals born and reared in isolation. Innate behavioral differences between the sexes can vary between species, are relatively invariant among conspecifics, and have likely evolved by natural or sexual selection.

If not from experience, where do innate sexual dimorphisms and differences in behavior come from? Our modern view of this problem arguably began with an article published in 1959 (Phoenix et al., 1959). Charles Phoenix and his colleagues were studying the mating behaviors of male and female guinea pigs. They unexpectedly discovered that when females were exposed to the male sex hormone, testosterone, before birth, they attempted to mate like males and lacked the normal copulatory behaviors of females. This result was a watershed in the field. The prevailing idea at the time was that sex hormones like testosterone acted only well *after* birth to trigger the expression of male-typical behaviors that were arranged earlier in life by genes and experience. The results of the experiment suggested, however, that testosterone was a male-specific developmental "switch" for sexual behavior—that is, it somehow functioned prenatally to developmentally organize the capacity for male behaviors that it also activated later in life.

In the sixty years that followed these observations, we have seen extensive evidence supporting and expanding Phoenix et al.'s main conclusions. In mammals, a male-specific surge of testosterone from the testes masculinizes discrete regions of the nervous system during a critical developmental window around the time of birth (Yang and Shah, 2014). The sexual differentiation of these regions is important in creating the neural potential for various sex dimorphisms and differences in the behaviors of juveniles and adults. Moreover, testosterone and its metabolite, estrogen, have substantial effects on the developing male rodent brain, sex-specifically regulating processes such as neurogenesis and cell death, neural differentiation, synaptogenesis and connectivity (Juntti et al., 2008; Juraska et al., 2013; McCarthy, 2020; Wu and Shah, 2011).

Studies in flies and worms have revealed similar phenomena. Although the dimorphic innate behaviors of flies and worms are not organized by gonadal hormones, they emerge from the largely cell-autonomous actions of key sex-determining regulatory genes during

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development. In the fruit fly *Drosophila melanogaster*, the sex-specific expression of transcription factors encoded by the *fruitless* and *doublesex* genes generates anatomical sex differences in neural circuits that underlie the dimorphic behaviors of adults (Billeter et al., 2006). Likewise, the dimorphic behaviors of the nematode *Caenorhabditis* (*C.*) *elegans* and the sexual differentiation of their nervous system is specified by the sex-specific expression of a transcription factor encoded by the *tra-1* gene (Portman, 2007). In all three animal systems, key sex-specific factors—*i.e.*, hormones or regulatory proteins—act during a critical developmental time to establish sex dimorphisms and differences in various regions of the nervous system that subserve the sex-biased behaviors displayed later in life.

In the last decade, how these factors implement the sexual differentiation of behavior and nervous systems has been elucidated in remarkable detail at molecular and cellular levels. Here, we bring to light these advances with case-studies of three animal systems. We first discuss mate recognition in *Drosophila* males and the neural circuit mechanisms that underlie their perception of sex pheromones. These neural pathways are almost entirely composed of neurons that are sexually dimorphic in anatomy. Focusing on one group of dimorphic neurons, the mAL cluster, we examine recently uncovered molecular and developmental processes through which the *fruitless* gene builds dimorphic neuroanatomy.

We then explore the sexual maturation of mating behaviors in *C. elegans*. We discuss a dimorphically connected neural circuit that serves a specific component of the adult male's mating behavior. Remarkably, this dimorphic circuit develops during sexual maturation from the rewiring of neurons that control a sexually monomorphic behavior during larval life. We examine recent studies that uncover the molecular mechanisms through which *tra-1* dimorphically rewires this circuit.

Finally, we turn to mammals and discuss how the release of testosterone during perinatal development specifies the willingness of juvenile male rats to play fight more than females. Recent studies have unveiled exciting connections between testosterone signaling, the endocannabinoid system, and microglia in the sexual differentiation of the amygdala and juvenile play fighting.

Taken together, these case-studies (1) illustrate the diversity of mechanisms that hormones and regulatory proteins use to sexually differentiate the nervous systems and behaviors of animals; (2) reveal mechanistic commonalities and differences among the three animal systems; and (3) highlight exciting future questions in the field.

2. Sexual differentiation of neurons regulating mate recognition in flies

Sexually reproducing animals are often confronted with a variety of potential mates, yet they have the impressive ability to choose mates that are suitable for successful reproduction. In *Drosophila*, males perform a complex courtship ritual toward conspecific females, whereas females decide whether to mate. The ability of *Drosophila* males to identify conspecific females is conveyed in part by waxy pheromones that coat the bodies of flies (Bontonou and Wicker-Thomas, 2014). When a male confronts a potential mate, he taps the fly's abdomen with a foreleg. Depending on what he tastes, he can tell if the fly is a male or a female of his species and offer his courtship only toward appropriate individuals.

In *Drosophila melanogaster*, the male's preference to court a conspecific female instead of a male is controlled in part by two contact-dependent pheromones called 7-Tricosene (7 T) and 7,11-Heptacosadiene (7,11HD) (Jallon, 1984). 7 T is only synthesized by males and suppresses male courtship. 7,11HD is a potent female-specific aphrodisiac that drives courtship (Billeter et al., 2009). Over the past 15 years, studies from several labs have described neural pathways involved in sensing and processing 7 T and 7,11HD. Remarkably, most elements of this circuit, from sensory to central neurons, are sexually dimorphic in anatomy, creating a male-specific substrate through which 7 T and 7,11HD regulate male courtship. Furthermore, the molecular mechanisms governing the sexual differentiation of one set of neurons within this circuit, the mAL neurons, have been elucidated in great detail.

2.1. The neural circuitry that perceives 7 T and 7,11HD in Drosophila males

7 T and 7,11HD are detected by gustatory sensory bristles on the male's foreleg when he taps a fly's abdomen early during courtship (Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012). Many of these sensory bristles house two gustatory sensory neurons that express a sodium channel gene called *pickpocket 23* (*ppk23*). One of the two *ppk23*-expressing sensory neurons, called the f-cell, responds selectively to 7, 11HD on females. Male-specific 7 T is detected by the other gustatory sensory neuron, called the m-cell (Thistle et al., 2012).

The neural pathways downstream of the f- and m-cells converge upon a group of male-specific courtship-promoting neurons in the posterior brain called P1 neurons (Kimura et al., 2008; Kohatsu et al., 2011). Upon pheromone input, the f-cells and m-cells promote and inhibit the activity of the P1 neurons, respectively, thereby producing opposite effects on male courtship. The neural pathways linking the f-cells and m-cells to the P1 neurons have been deciphered (Fig. 1a) (Clowney et al., 2015; Kallman et al., 2015). The f-cells send axons into the ventral nerve cord of the fly (analogous to our spinal cord), where they provide input to two excitatory ascending neuronal types, vAB3 (Clowney et al., 2015) and PPN1 (Kallman et al., 2015). Both neural types innervate the lateral protocerebral complex in the brain, where dendrites of the P1 neurons reside.

Like the f-cells, the m-cells send afferents into the ventral nerve cord, but they also project ascending neurites to the subesophageal zone located ventrally in the central brain (Kallman et al., 2015; Lu et al., 2012; Thistle et al., 2012). There, the m-cells connect to fibers from a group of GABAergic inhibitory interneurons called the mAL neurons (Fig. 1a) (Kallman et al., 2015). The mALs project axons to the lateral protocerebral complex and provide inhibitory input to the P1 neurons (Clowney et al., 2015; Kallman et al., 2015). Thus, whereas the f-cells provide the courtship-promoting P1 neurons with feedforward excitation through a disynaptic pathway, the m-cells block courtship drive by suppressing the activation of P1 neurons through GABAergic mAL neurons.

Of these circuit elements, all but one (PPN1), are sexually dimorphic in anatomy. For instance, some components, like the f- and m-cells (Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012), and the mAL (Kimura et al., 2005) and P1 neurons (Kimura et al., 2008), display sex differences in neuron number, whereas others, like the vAB3 neuron (Von Philipsborn et al., 2014; Yu et al., 2010) and some mAL neurons (Kimura et al., 2005), are present in both sexes but exhibit dimorphic arborizations or projections. Although the function of these circuit elements in females is not entirely clear, the sex differences in neuroanatomy are likely to be important in establishing the function and connectivity of the neural circuit that mediates responses to 7 T and 7,11HD in males. But how do these sex differences develop? Our most detailed view into this problem has come from studies on the mAL neurons.

2.2. The sexually dimorphic mAL neurons

The mAL neurons ("neurons medially located, just above antennal lobe; " Lee et al., 2000) (Fig. 1b) are a bilateral group of approximately thirty neurons in the male brain and five neurons in the female brain (Kimura et al., 2005). In both sexes, the cell bodies of the mAL neurons are located just above the antennal lobes and extend bundled neurites contralaterally. Upon crossing the midline, the neurites bifurcate to form a dorsal branch that innervates the lateral protocerebral complex, providing inputs to the P1 neurons; and a ventral branch that extends into the subesophageal zone, where in males they receive input from the m-cells and vAB3 neuron (Clowney et al., 2015; Kallman et al., 2015). Aside from differences in cell number, the mAL neurons display other

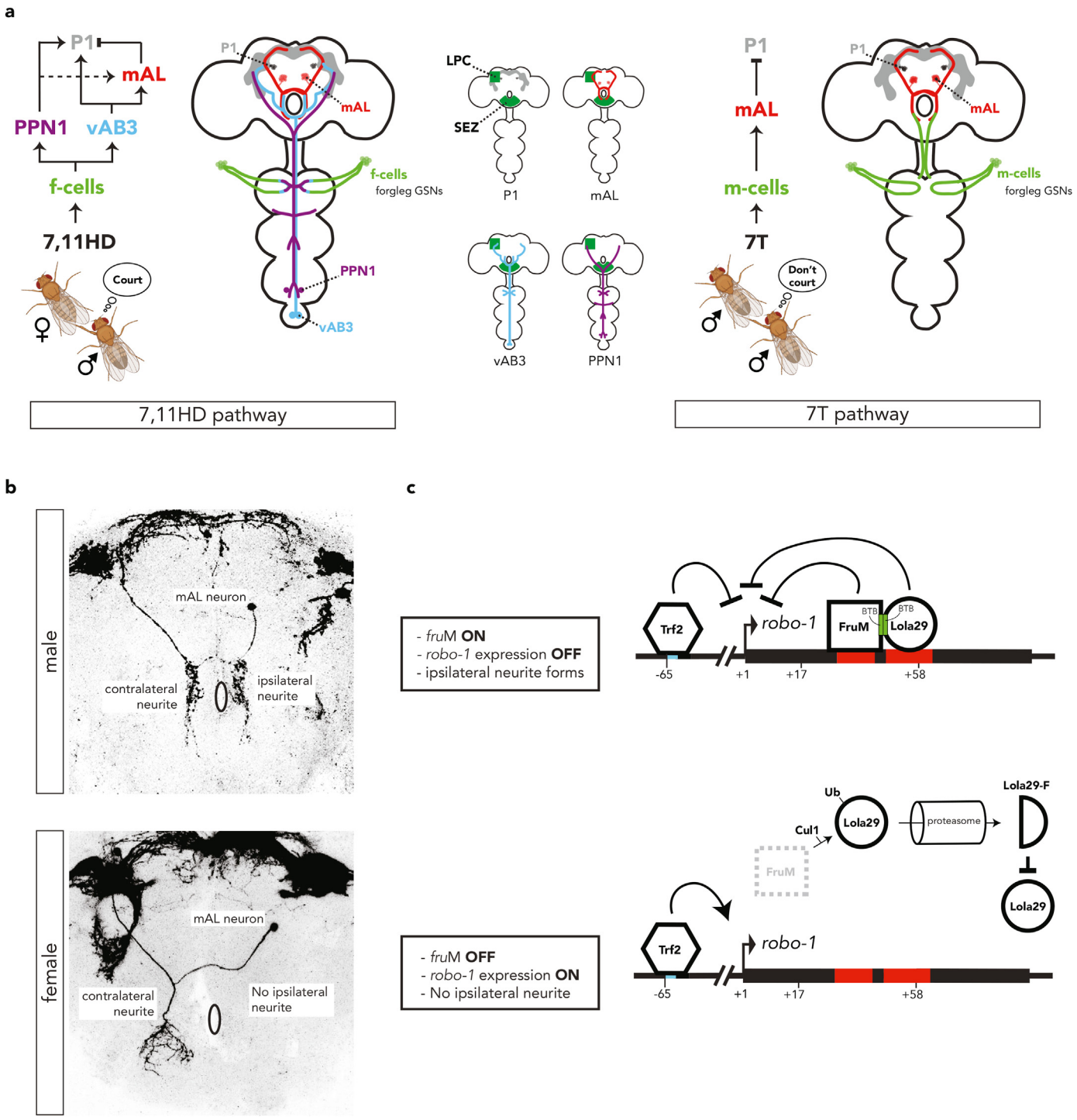

Fig. 1. Neural pathways of mate recognition in *Drosophila* males and the dimorphic development of the mAL neurons. (a) Models of the neural pathway downstream of the female-specific aphrodisiac 7,11HD (left) and the male-specific anti-aphrodisiac 7 T (right). Illustrations of the brain and ventral nerve cord show the relative positions and projection patterns of the neurons under study. In the 7,11HD pathway, the GABAergic mAL neurons are activated by the PPN1 and vAB3 neurons and inhibit the activity of the P1 neurons (Clowney et al., 2015; Kallman et al., 2015). PPN1 most likely activates the mAL neurons indirectly through another currently unidentified neuron(s). This prevents the excitation of the P1 neurons from getting saturated in response to PPN1 and vAB3 input, thereby keeping the P1 neurons sensitive to courtship cues (Clowney et al., 2015). Illustrations were adapted from Clowney et al. (2015). Illustration of flies from Biorender. (b) A single GFP-labeled mAL neuron on one side of the brain is shown for the male (top) and female (bottom). Additional neurons are also targeted as a result of the labeling method. Images kindly provided by K. Sato and D. Yamamoto (Tohoku University). (c) An illustration of the mechanisms by which FruM protein regulates the transcription of *robo-1* and the formation of the mAL neurons' male-specific ipsilateral neurite.

sexual dimorphisms. In addition to the contralateral branch, most mAL neurons in males extend a second branch into the subesophageal zone on the ipsilateral side. This branch is missing from all mAL neurons in females. How the mAL neurons contribute to female behavior and how these anatomical dimorphisms influence circuit function are currently unclear.

During development, a male-like number of mAL neurons are initially born in both sexes from a common neuronal stem cell, but a subset

undergoes apoptosis in the female (Kimura et al., 2005). The thirty mAL neurons in the male and the remaining five in the female then undergo male- or female-type differentiation in the anatomy of their neurites. Recent studies from Daisuke Yamamoto's group have provided an exceptional view into the molecular mechanisms governing the dimorphic development of the mAL neurons (Sato and Yamamoto, 2020), most notably in the formation of the male-specific ipsilateral branch.

2.3. Why the mAL neurons extend an ipsilateral neurite in males but not in females

During neuronal development, the extension of a neurite is regulated by receptor-ligand interactions that mediate attraction or repulsion toward specific targets. The formation of the mAL neurons' ipsilateral branch is controlled by an immunoglobulin-type cell adhesion receptor encoded by the *roundabout-1* (*robo-1*) gene (Ito et al., 2016). *Robo-1* is expressed in some or all female mAL neurons where it suppresses formation of the ipsilateral branch; in males, *robo-1* transcription is blocked, and the extension of the ipsilateral neurite is disinhibited.

The dimorphic expression of *robo-1* is regulated during pupal development by a key male-specifically-expressed BTB/zinc-finger transcriptional regulator called *fruitless* (*fru*M) (Dalton et al., 2013; Ito et al., 2016; Neville et al., 2014; Vernes, 2014). Expression of *fru*M protein occurs in the mAL neurons of males likely right after neuronal birth where it blocks cell death and directs male-specific neuronal differentiation (Kimura et al., 2005). *Fru*M promotes the male-specific formation of the ipsilateral branch by binding to a *cis*-regulatory element near *robo-1*'s promoter and directly repressing *robo-1* transcription (Fig. 1c) (Ito et al., 2016). *Fru*M acts by converting a core promoter recognition factor called *Trf2* from a transcriptional activator to a repressor (Chowdhury et al., 2017). In females, the absence of *fru*M derepresses *robo-1* transcription, and the expression of *robo-1* inhibits the formation of the ipsilateral branch (Ito et al., 2016).

More recently, Yamamoto's group discovered an exciting new twist into how *fru*M represses *robo-1* expression (Sato et al., 2019). It turns out that an isoform of the *longitudinals lacking* (*lola*) gene, *lola-29*, is also required to repress *robo-1* expression in the mAL neurons of males (Sato et al., 2019). Similar to *fru*M, *lola-29* encodes a BTB/zinc-finger transcription factor (Zhang et al., 2003). It binds to a *cis*-regulatory element in the *robo-1* locus immediately adjacent to *fru*M's binding site (Sato et al., 2019) and contributes to suppression of *robo-1* expression (Fig. 1c). Intriguingly, females normally express a truncated version of *lola-29*, *lola-29-F*, along with the full-length isoform found in males (Sato et al., 2019). *Lola-29-F* is generated by a ubiquitin/proteasome-mediated process that partially degrades the N-terminal portion of the full-length protein, including *lola-29*'s BTB domain (Sato et al., 2019). The female-specific, truncated *lola-29* interferes with the function of the full-length protein, thereby relieving *lola-29*-mediated repression of *robo-1* in females but not males (Sato et al., 2019).

Why is *lola-29* proteolytically processed in females but not males? The answer, surprisingly, turns out to be *fru*M. Biochemical experiments suggest that *fru*M somehow shields *lola-29* from degradation most likely through protein-protein interactions via their respective BTB domains (Sato et al., 2019). In females, because *fru*M is not expressed, *lola-29* is unprotected and proteolytically processed. Thus, in addition to its role in directly regulating *robo-1* transcription, *fru*M influences dimorphic development by regulating the proteolysis of *lola-29*.

Taken together, mate recognition in *Drosophila* males is controlled by anatomically dimorphic feedforward excitatory and inhibitory neural pathways that are tuned to female- or male-derived pheromones, respectively. Studies on the *fruitless* gene and the mAL neurons have illustrated how a sex-determining transcriptional regulator may pattern sexually dimorphic neuronal traits in this neural circuit. These works have revealed an unexpected and exciting link between *fruitless*-regulated transcription, sex-specific proteolysis, and the development of a neuronal dimorphism.

3. Sexual maturation of mating behavior in worms

Animals undergo numerous changes in behavior when they transition from immaturity to adulthood, the most dramatic of which is the emergence of sexual dimorphisms and differences in behaviors for reproduction. Sexually inactive juveniles with few sex differences transform into adults with complex social and mating behaviors that are strikingly divergent between the sexes. How do neural circuits for these behaviors arise during maturation?

Studies on the nematode *Caenorhabditis* (*C.*) *elegans* have offered exciting new insights into this question. *C. elegans* exist as two sexes—a male and a sperm-producing female called a hermaphrodite. Adult male and hermaphrodite worms display obvious dimorphisms in mating behavior. The neural potential for these behaviors develops when the worm sexually matures late in its juvenile larval life just before adulthood.

Like some other animals, much of the neural circuitry for dimorphic behaviors in worms is thought to develop during sexual maturation from sex-specific neurons that wire into an existing sex-shared nervous system. However, recent work in *C. elegans* tells us that the neural circuitry for dimorphic behaviors is also composed of sexually dimorphic neurons present in both sexes that were monomorphic and active in juvenile worms. The molecular and developmental mechanisms that repurpose these sex-shared neurons in juvenile worms for dimorphic functions in the adult are becoming illuminated in great detail.

3.1. A dimorphically connected neural circuit regulates a sex-specific behavior in the adult worm

When a *C. elegans* male attempts to mate with a hermaphrodite (Fig. 2a) (Liu and Sternberg, 1995), he attaches the ventral side of his tail to her and begins to move backwards along the length of her body. Once he reaches her head or tail, he arches his tail, turns, and continues to move on the other side of her until he nears the vulva. There, in response to hermaphrodite-derived signals, he stops, extends a pair of copulatory structures called spicules, inserts them into the hermaphrodite's vulva, and proceeds to transfer sperm.

The neural mechanisms that control the male's copulatory behavior have been investigated recently. The adult male's tail contains a variety of sensory structures, two of which—the hook and the bilaterally paired phasmids—are important in detecting cues when the male contacts a hermaphrodite and attempts to locate the vulva (Liu and Sternberg, 1995; Oren-Suissa et al., 2016). Although the hook is specific to males, the phasmids are present in both sexes and have an altogether different function in the adult hermaphrodite. The phasmids are innervated by two single bilaterally paired chemosensory neurons, PHA and PHB, that, in the adult hermaphrodite, modulate avoidance behaviors in response to chemorepellents (Hilliard et al., 2002). Interestingly, in the adult male, the PHB neurons do not appear to contribute to chemorepulsive processing but mediate his response to contacting a hermaphrodite and function to locate the hermaphrodite vulva during mating behavior (Oren-Suissa et al., 2016).

The sex differences in phasmid sensory neuron function are associated with sex differences in their neuronal connectivity with postsynaptic cells. The nervous system of adult *C. elegans* consists of 294 neurons shared between the sexes that are integrated with 8 hermaphrodite-specific or 93 male-specific neurons (Barr et al., 2018; Emmons, 2018; Molina-García et al., 2020). Although dimorphic behaviors in *C. elegans* are regulated in large part by the sex-specific neurons, a number of the sex-shared neurons have been found to possess sexual dimorphisms (Portman, 2017) in function (Fagan et al., 2018; Jang et al., 2012; Lee and Portman, 2007; Wan et al., 2019; White et al., 2007), gene expression (Lee and Portman, 2007; Serrano-Saiz et al., 2017), morphology (Hart and Hobert, 2018; Serrano-Saiz et al., 2017), and notably, synaptic connectivity (Oren-Suissa et al., 2016). In comparing EM-based reconstructions of the connectome within the posterior nervous system of

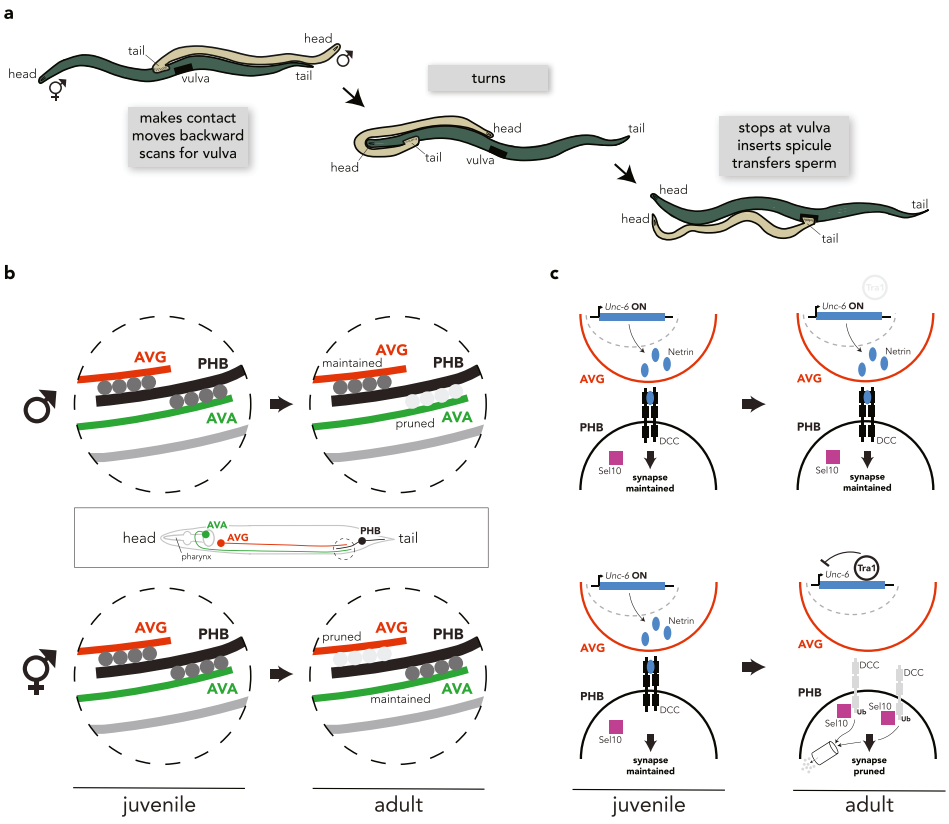

Fig. 2. *C. elegans* mating behavior and the sexually dimorphic rewiring of synapses during sexual maturation. (a) Illustration of the innate mating behaviors of *C. elegans* males. The phasmid sensory neurons are located in the male and hermaphrodite tail and are used by the male to locate and stop near the hermaphrodite vulva. Illustration adapted from Emmons (2018). (b) Reconfiguration of PHB-AVA and PHB-AVG synapses during sexual maturation. PHB's synapses with AVG and AVA are maintained and pruned, respectively, in adult males. Conversely, synapses between PHB and AVG, and PHB and AVA are pruned and maintained, respectively, in adult hermaphrodites. Illustration adapted from Oren--Suissa et al. (2016) and Slazberg et al. (2020). (c) The molecular pathway that regulates synaptic connectivity between PHB and AVG in males and hermaphrodites.

males and hermaphrodites, several of the sex-shared neurons were discovered to exhibit striking sexual differences in their patterns of connectivity (Cook et al., 2019; Jarrell et al., 2012; White et al., 1986). The PHB phasmid sensory neuron, for instance, innervates completely different target interneurons in adults of the two sexes (Oren-Suissa et al., 2016) (Fig. 2b). In hermaphrodites, PHB forms synaptic connections with several neurons including an interneuron called AVA. Although AVA is present in males, the PHB neuron instead innervates an interneuron called AVG and other neurons common to both sexes. These two circuit configurations account for the differences in PHB-mediated behaviors between adult males and hermaphrodites.

3.2. Reconfiguring PHB's synapses during sexual maturation

In *C. elegans*, the neural circuits for dimorphic reproductive behaviors are assembled during sexual maturation in the final larval stage prior to adulthood. The PHB, AVA, and AVG neurons are all born during embryogenesis and exist throughout larval life (Oren-Suissa et al., 2016; Sulston et al., 1983). It was thus conceivable that PHB could form its synaptic connections with AVA in the hermaphrodite and AVG in the male *de novo* during sexual maturation. However, analyses of PHB's synaptic connectivity throughout the larval stages revealed that PHB is in fact connected to AVA *and* AVG in *both* sexes well before sexual maturation (Fig. 2b) (Oren-Suissa et al., 2016). Remarkably, PHB's connectivity with AVA and AVG becomes sex-specific in the adult as a result of synaptic pruning and maintenance mechanisms that eliminate and preserve PHB's synapses with either AVA or AVG depending upon the sex of the worm (Oren-Suissa et al., 2016).

Thus, the neural circuits underlying sexually dimorphic behaviors in the worm arise not only from the gain of sex-specific neurons that wire into a shared nervous system, but also from the synaptic reconfiguration of pre-existing circuit elements that influence sexually monomorphic behaviors in the juvenile.

The molecular mechanisms that maintain synapses between PHB and AVG in the male has received attention recently (Fig. 2c). Two key players are a widely conserved secreted axon guidance protein called Netrin and its receptor DCC, which in *C. elegans*, are encoded by the *unc-6* and *unc-40* genes, respectively. Activity of the DCC receptor is necessary to maintain synaptic contacts between the PHB and AVG neurons in males (Weinberg et al., 2018). DCC is present on the surface of the PHB neuron of both sexes through early larval stages, but then disappears from the PHB neuron of hermaphrodites at around the time of sexual maturation (Salzberg et al., 2020; Weinberg et al., 2018). As a result, the PHB-AVG connection gets pruned in hermaphrodites but not in males.

A new study from Meital Oren-Suissa's group (Salzberg et al., 2020) demonstrated that expression of DCC protein in the PHB neuron is regulated by the ubiquitin/proteasome pathway (Fig. 2c). The FBW-7 E3 ubiquitin ligase, encoded by the *sel-10* gene in worms, targets the DCC receptor for degradation only in hermaphrodites. This appears to happen specifically in hermaphrodites due to Netrin. Netrin is expressed in the AVG neuron of both sexes in young larvae, but then becomes restricted to males near the onset of sexual maturation (Weinberg et al., 2018). Intriguingly, genetic epistasis experiments suggest that the release of Netrin somehow prevents FBW-7-mediated degradation of DCC in the PHB neuron (Salzberg et al., 2020). Furthermore, DCC can promote the maintenance of PHB-AVG synapses even in the absence of Netrin signaling—as long as the presence of DCC protein on PHB's surface is stabilized (Salzberg et al., 2020). Thus, Netrin functions primarily to stabilize DCC by "protecting it" from protein degradation, thereby causing DCC-dependent synaptic maintenance. As the worm enters maturation, Netrin expression declines in the AVG neuron of hermaphrodites; DCC gets degraded in the PHB neuron; and the synaptic connections between PHB and AVG are then pruned.

What restricts Netrin expression to the AVG neuron of males, and how is Netrin's switch from monomorphic to dimorphic expression scheduled to occur late in larval life? In *C. elegans*, sexual differentiation is controlled by a *Gli/Cubitus Interruptus*-like Zn-finger transcription factor called *tra-1* (Hodgkin, 1987; Zarkower and Hodgkin, 1992). *Tra-1* is

expressed in XX worms and promotes hermaphrodite development of the soma and nervous system cell-autonomously by regulating cell-type-specific effectors. In *XO* worms, *tra-1* is largely off and male somatic development occurs by default (Bayer et al., 2020; Lawson et al., 2020; Schvarzstein and Spence, 2006; Starostina et al., 2007). *Tra-1* protein in the hermaphrodite nervous system accumulates over the course of larval development and becomes broadly expressed in post-mitotic neurons, including the AVG neuron, at around the start of sexual maturation (Bayer et al., 2020; Lawson et al., 2020; Weinberg et al., 2018). In the AVG neuron of hermaphrodites, *tra-1* binds to *cis*--regulatory sequences within the *unc-6* gene where it transcriptionally represses Netrin expression (Fig. 2c) (Weinberg et al., 2018). Thus, the timing of Netrin's dimorphic expression may be an indirect result from the onset of *tra-1* expression in the AVG neuron in hermaphrodites.

How the timing of *tra-1* expression in the hermaphrodite nervous system is regulated is currently unclear, but a recent study has provided some clues. The timing of developmental events in the worm is controlled by a phylogenetically conserved genetic pathway of transcription factors, RNA-binding proteins and microRNAs (Ambros, 2000; Rougvie and Moss, 2013). This pathway of genes—dubbed the 'heterochronic pathway'— also schedules the sexual maturation of the worm's nervous system (Lawson et al., 2019; Pereira et al., 2019). Interestingly, a nuclear hormone receptor called DAF-12 that acts downstream of the heterochronic pathway (Antebi et al., 2000) was recently found to upregulate *tra-1* expression in the hermaphrodite nervous system as the worm approaches sexual maturation (Bayer et al., 2020). This finding is particularly exciting as it potentially implicates hormonal signaling in the regulation of sexual identity and maturation in the worm.

Studies in *C. elegans* have given deep insights into the mechanisms by which circuits for dimorphic adult behaviors arise. In addition to sex-specific neurons, these circuits are composed of neurons that are repurposed during maturation from circuits that influence monomorphic behaviors in the juvenile. Recent work has put the spotlight on the ubiquitin/proteasome system and its function in sex-specifically rewiring juvenile neurons for use in mating behaviors of the adult.

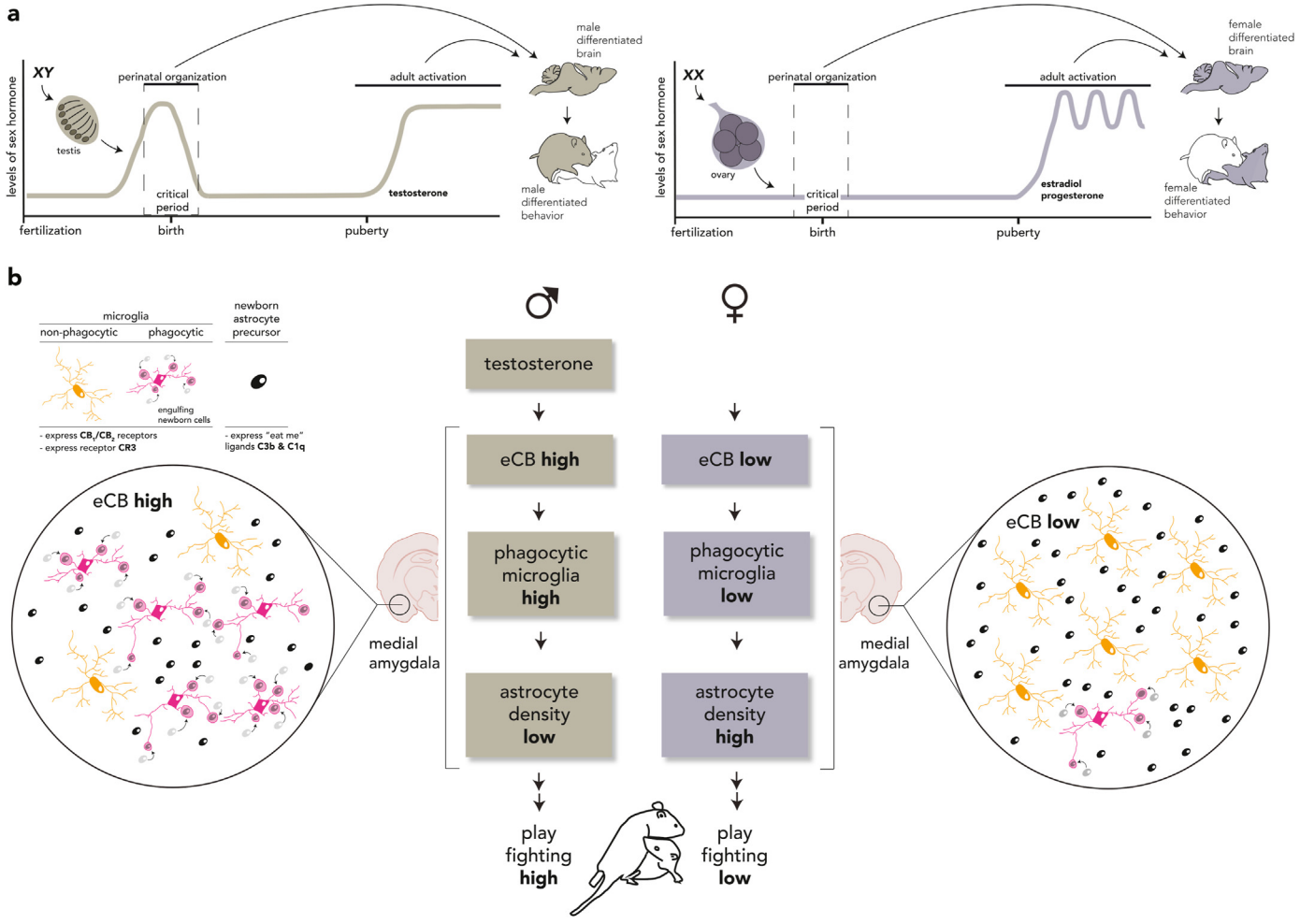

Fig. 3. Sexual differentiation of behavior and nervous systems in mammals and the development of dimorphic play fighting in juvenile rodents. (a) Sexual differentiation of behavior and nervous systems in mammals. (Left) In *XY* males, the testes release testosterone during a critical, developmentally sensitive perinatal period. The critical period ends when female behavior can no longer be masculinized/defeminized by administering testosterone. Testosterone acts through the androgen receptor to masculinize and defeminize specific regions of the brain that subserve sex differences in juvenile (*e.g.*, play fighting) and adult behaviors (*e.g.*, sexual behaviors; shown) and physiological processes. In rodents, testosterone also gets converted to estradiol locally in the brain by region-specific expression of the *aromatase* gene. Estrogen acts through estrogen receptors independent of testosterone to male-specifically regulate a variety of neuro-developmental processes such as apoptosis, neurogenesis, and synaptogenesis. Note, there is no evidence for estrogen in the masculinization of the primate brain. At around the time of puberty, the testes recommence a steady-state release of testosterone which primarily acts to activate sex-specific adult behaviors (like mating) that were developmentally organized during perinatal life. (Right) In *XX* females, the ovaries are quiescent through early life, and the brain and behavior feminize by default. Around puberty, the ovaries begin to release sex hormones (*e.g.*, estradiol and progesterone) in cyclical manner (as a result of ovulation) that act to regulate the activation of adult-specific female behaviors. (b) The molecular and cellular pathway that regulates the dimorphic potential for juvenile play fighting in rodents. Brain illustration from Biorender. See text for details.

4. The development of sex differences in the play of young rodents

The young of many mammals like to play fight. They take turns playfully attacking each other with aggressive or even mating-like tactics but do so with restraint so that it seldom becomes serious. Play fighting during juvenile life is thought to facilitate the proper development of social behaviors like aggression or mating in adulthood (Pellis and Pellis, 2017). The way animals play fight varies across species, but as a general rule, juvenile males play fight more than females (Pellis et al., 1997).

When domesticated rats play fight as juveniles, the male attacks the nape of his playmates' neck and attempts to nuzzle his snout in it through a sequence of complex behaviors. These behaviors occur more frequently among males than in females and are similar to those of adult male rats when they attempt to mate with females. In the past ten years, the developmental and molecular processes that incline young male rats to play fight more than females have been investigated. These studies reveal exciting roles for the endocannabinoid system and for immune cells of the brain called microglia, both acting downstream of gonadal sex hormones, in creating a sex difference in the potential for play fighting in young rats.

4.1. Testosterone masculinizes play fighting through endocannabinoids

Sexual differentiation of the nervous system and behavior in rodents occurs in large part during a critical period around the time of birth (Fig. 3a) (Juraska et al., 2013; McCarthy, 2020; McCarthy et al, 2017, 2018; Wu and Shah, 2011). Steroid hormones like testosterone get released from the fetal testes just before birth and enter the nervous system where they masculinize different regions of the brain over the course of a few days through the time of birth. In females, the ovaries are quiescent during this time and female differentiation of the nervous system occurs due to the absence of testosterone. In males, testosterone acts through the androgen receptor, but in some mammals like mice, it can also masculinize the nervous system by getting converted to estrogen in the brain and signaling through estrogen receptors (Wu and Shah, 2011). The male-specific surge of circulating testosterone during perinatal life regulates neurodevelopmental processes such as neurogenesis, neuronal death, and synaptogenesis, in turn generating neuroanatomical dimorphisms and quantitative differences that are important for male behaviors.

The higher inclination of young males to play fight is built by the surge of testosterone during perinatal life. Studies in the 1980s showed that females can exhibit male-like levels of play fighting as juveniles if they received testosterone at the time of birth (Meaney and Stewart, 1981). Conversely, juvenile males lacking androgen receptor function display reduced levels of play fighting comparable to those of normal females (Meaney et al., 1983). Lesions in the amygdala of juvenile rats also suppress play fighting in males to female-like amounts (Meaney et al., 1981), indicating that the amygdala is important in regulating the sex difference in play-fighting levels, but not the ability to play-fight per se. Indeed, other regions of the rat brain are critical contributors to play-fighting behavior (Beatty et al., 1982; Beatty and Costello, 1983; Bell et al., 2009; Kamitakahara et al., 2007). Together, these observations led to the hypothesis that testosterone-dependent sexual differentiation of the amygdala during the perinatal period is critical in establishing a sex difference in levels of juvenile play fighting. Accordingly, introducing testosterone directly into the medial amygdala of female pups is sufficient to induce male-like amounts of play behavior in juvenile females (Meaney and McEwen, 1986).

What kinds of sex differences in the amygdala could underlie sex differences in juvenile play fighting? And by what cellular and molecular mechanisms does testosterone implement those sex differences? The amygdala influences numerous sexually dimorphic social behaviors from mating, aggression and territoriality to emotional memory and parental care (Hamann, 2005; Newman, 1999). Not surprisingly, the amygdala contains a variety of sex differences in features such as cell number, and dendritic and synaptic morphology (Johnson et al, 2008, 2012, 2013). Clues into the types of differences in the amygdala that may contribute to the higher levels of play fighting in males initially emerged from studies by Margaret McCarthy's laboratory on the connection between the endocannabinoid system and play fighting.

Endocannabinoids (eCBs) (Harkany et al., 2007) are endogenous ligands—primarily N-arachidonylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG)—that act through two G protein-coupled cannabinoid receptors, CB_1 and CB_2. eCB signaling in the mammalian brain is pleiotropic, regulating synaptic activity, postnatally, and numerous neurodevelopmental processes, prenatally. Interestingly, when female rats were given a non-selective cannabinoid receptor agonist during the developmentally sensitive perinatal period, they later exhibited male-like levels of play-fighting as juveniles (Krebs-Kraft et al., 2010). Thus, the activity of the endocannabinoid system during perinatal life in males is important in building the sex difference in the potential for juvenile play-fighting.

Correlated with the effect on play fighting, eCB signaling also appears to regulate a sex difference in the number of newborn cells in the medial amygdala of neonatal pups, wherein females have more newborn cells than males (Krebs-Kraft et al., 2010). These additional newborn cells in female pups are precursor cells that primarily give rise to metabolic and supportive cells of the nervous system called astrocytes (Krebs-Kraft et al., 2010), thereby supplying juvenile females with more astrocytes in the medial amygdala than males. Notably, the sex difference in the number of newborn cells is dependent upon eCB signaling; activation of cannabinoid receptors in neonatal females decreases the number of newborn cells and astrocytes to an amount that is similar to males (Argue et al., 2017; Krebs-Kraft et al., 2010). Furthermore, the medial amygdala of neonatal males contains higher amounts of 2-AG than females, which is dependent upon testosterone signaling via the androgen receptor (Krebs-Kraft et al., 2010).

These data suggest that higher eCB signaling in males reduces the number of newborn astrocyte precursors in the medial amygdala (Fig. 3b). The smaller number of astrocytes somehow causes a higher level of play-fighting in juvenile males than in females. It is noteworthy that when juveniles are partnered with a playmate, the neural activity in the medial amygdala is higher in males than in females (VanRyzin et al., 2019). Developmental manipulations that increase astrocyte density in the medial amygdala of males cause a corresponding reduction in neural excitation and levels of play-fighting (VanRyzin et al., 2019). Thus, the higher density of astrocytes in the medial amygdala of females may reduce neural activity, thereby causing a reduced propensity for females to play fight as juveniles.

4.2. Microglia mediate the effects of eCB signaling on newborn cell number in males

Nearly ten years after revealing a link between the eCB system and play fighting in male rats, McCarthy's group discovered the mechanism by which eCB signaling causes a reduction in newborn cell number in the medial amygdala of males (Fig. 3b) (VanRyzin et al., 2019). The key player turned out to be microglia. Microglia are the innate immune cells of the brain similar to macrophages. They originate early in embryonic development from precursors in the yolk-sac that migrate into the developing embryonic neural tube. Once in the embryonic brain, the precursor cells continue to proliferate, differentiate and mature into microglia, and permanently reside in the brain. Although best known for their role in phagocytosing dead or dying cells during the brain's response to infection or injury, microglia also actively mediate a variety of processes that occur during normal brain development such as neurogenesis, axonal and synaptic development, and neural connectivity (Bordt et al., 2020; Lenz and McCarthy, 2015; Nelson et al., 2019).

In the medial amygdala of newborn rats, males and females have an equivalent number of microglia but males have a greater percentage that

are phagocytic (VanRyzin et al., 2019), *i.e.,* engaged in the act of eating other cells. The sex difference in the frequency of phagocytic microglia is regulated by testosterone and eCB signaling through the CB_1 and CB_2 receptors, both of which are expressed in microglia (Stella, 2009; VanRyzin et al., 2019). Remarkably, the microglia are primarily eating newborn cells that were alive and destined to become—you guessed it—astrocytes. Blocking the function of these microglia in the medial amygdala of males increases the number of astrocyte-fated newborn cells and reduces the level of juvenile play-fighting to amounts comparable in females. The ability of microglia to recognize and preferentially eat newborn cells is controlled by receptor-ligand interactions of the complement system. The microglia express a complement system receptor called CR3, which recognizes two ligands, C1q and C3, both of which are enriched on the surface of newborn cells in the amygdala (VanRyzin et al., 2019).

Taken together, the available data supports the following model (Fig. 3b): the proclivity of young male rats to play fight more than females is specified during perinatal life when a surge of testosterone from the testes acts through the androgen receptor to upregulate local eCB content in the medial amygdala of males relative to females. The higher amounts of eCB signaling recruits more microglia to phagocytose newborn, astrocyte-fated cells in a complement-dependent manner. As a result, the medial amygdala of males develops with fewer astrocytes, leading to greater neural excitability in the amygdala and a higher propensity for males to play fight as juveniles. The cellular source of the eCBs, how eCB signaling induces phagocytic microglia, and how astrocytes regulate the neural circuits in the amygdala for play-fighting are all open questions.

This work adds to a large body of evidence that microglia, other immune cells, and inflammatory signals play central roles in implementing the effects of gonadal sex hormones on the sexual differentiation of the mammalian nervous system (Nelson et al., 2019). Indeed, sex differences in the number and morphology of microglia have been well documented within many regions of the rat brain including the preoptic area, amygdala, and hippocampus (McCarthy, 2020). How these differences contribute to the sexual development of the brain and behavior are active areas of research.

5. Conclusion

In speculating on the "nature of the modifications" produced by testosterone, Phoenix and his colleagues were doubtful that it was drastic or even anatomical and instead submitted that "a more subtle change reflected in function rather than in visible structure would be presumed." Although their paper was a landmark in the field, this particular hypothesis did not age well. We now know that sex hormones have considerable, region-specific developmental effects on the structure and connectivity of the mammalian brain, differentiating the brain and behavior in male- or female-like directions. Decades of studies in other animal systems have revealed a similar picture, if not for gonadal sex hormones, for key sex-determining regulatory genes.

The three case-studies explored here are textbook examples of how a "master" sex-specific hormone or regulatory protein establishes an innate sexual dimorphism or difference in behavior. We saw how the fly sexual differentiation gene, *fru*M, regulates transcription and proteolysis to control the formation of a male-specific neurite in neurons within a sex pheromone circuit. In worms, we learned how hermaphrodite-specific expression of *tra-1* regulates Netrin-DCC signaling to reconfigure the connectivity of a juvenile neural circuit for dimorphic functions in the adult. Finally, by regulating the endocannabinoid system and the activity of microglia, we saw how testosterone implements a sex difference in astrocyte cell number within the medial amygdala, thereby causing young male rats to play fight more than females.

5.1. Mechanistic similarities and differences across the three case-studies

In comparing these case-studies, a number of mechanistic commonalities and differences emerge. In all three cases, in the absence of sexual differentiation, neuronal and behavioral development occurs monomorphically, but in the likeness of one or the other sex. For instance, when testosterone is removed from perinatal male rats, their levels of play fighting as juveniles becomes fully female-like (Meaney et al., 1983); the mAL neurons of males are completely feminized in the absence of *fru*M activity (Kimura et al., 2005); and without *tra-1*, the synaptic connectivity between the PHB and AVA and AVG neurons in hermaphrodites develops as it does in males (Oren-Suissa et al., 2016). These examples suggest that some sex differences or dimorphisms in behavior are *primed* to develop as "male" or "female" in *both* sexes. A sex difference then arises when a sex-specific hormone or regulatory protein redirects this basal, sex-non-specific developmental potential toward the opposite sex.

Another mechanistic similarity across the three systems is the use of pruning, whether it be synapses or cells, to create a sex difference. The difference in astrocyte density in the medial amygdala of male and female rats develops from the male-specific removal of astrocyte precursors by microglia, triggered by perinatal testosterone and endocannabinoids (Krebs-Kraft et al., 2010; VanRyzin et al., 2019); *Drosophila* males develop a greater number of mAL neurons because the male-specific expression of *fru* maintains a set of neurons that would otherwise undergo cell death (Kimura et al., 2005); and in worms, the connectivity between the PHB, AVA and AVG neurons becomes dimorphic as a result of sex-specific synaptic pruning mechanisms (Oren-Suissa et al., 2016; Salzberg et al., 2020; Weinberg et al., 2018). In each case, a sex-specific effector specifies a sex difference in behavior by regulating a pruning mechanism that cuts back cells or synapses in one sex.

In rodents, sex differences in play fighting and other behaviors are established during a critical period around the time of birth soon after the onset of testosterone production. This phenomenon of "early life programming," in which long-lasting changes in the nervous system occur during a specific developmental period in anticipation of behaviors later in life (McCarthy et al, 2017, 2018; Phoenix et al., 1959), also influences the innate dimorphic behaviors of other animals. The courtship behaviors of male and female flies are programmed during a critical period that corresponds to around mid-pupal life (Arthur et al., 1998; Belote and Baker, 1987), as the nervous system transforms from its larval to adult form. Expression of male-specific *fru* in the brain peaks during this time (Lee et al., 2000) where, among many actions, it blocks neuronal cell death in the mAL cluster and directs the mAL neurons toward a male fate (Kimura et al., 2005). Similarly, in worms, the potential for dimorphic mating behaviors develops during a period of sexual maturation in late larval life when *tra-1* expression in the hermaphrodite nervous system peaks (Bayer et al., 2020; Lawson et al., 2020) and the dimorphic connectivity between PHB, AVA, AVG and other neurons is built (Oren-Suissa et al., 2016).

Aside from the downstream molecules and developmental events, there are also notable mechanistic differences in the three case-studies we discussed. Although the three sex-specific effectors are all expressed downstream of sex-determining mechanisms, they regulate development cell-autonomously or cell-non-autonomously. Testosterone is a signaling molecule released from the testes that gains access to the brain and regulates development in target cells through the androgen receptor; *fru*M and *tra-1* are transcription factors that regulate neuronal development autonomously, in the cells in which they are expressed. However, *fru*M and *tra-1* can also regulate sexual differentiation cell-non-autonomously. For instance, expression of *fru*M in certain abdominal motoneurons induces the male-specific development of an abdominal muscle (Gailey et al., 1991; Lawrence and Johnston, 1986; Usui-Aoki et al., 2000) and *tra-1* expression in the AVA neuron, for example, contributes to synaptic development between the PHB and AVG neurons (Oren-Suissa et al., 2016).

Taken together, the three case-studies discussed here illustrate a few common strategies that animals use to create an innate sex difference or dimorphism in behavior. Innate sex differences in behavior develop from

the actions of sex-specific hormones or regulatory proteins that direct the sexual differentiation of the animal's nervous system. These effectors act during a critical developmental window to induce enduring changes in discrete regions of the nervous system. These regions are primed to develop monomorphically and in the likeness of one sex. The sex-specific expression of the effector redirects development toward a path typical of the opposite sex. Although the downstream molecules and developmental endpoints of these effectors vary considerably across systems, they all tend to sex-specifically prune cells in the nervous system in various ways (*e.g.*, the cells themselves or synapses), thereby creating a substrate for a sex difference in behavior.

5.2. Looking ahead

One of the next challenges in the future is to understand how the neural circuits for dimorphic behaviors as a whole are molecularly assembled. Neurons exhibit molecular properties that allow them to form synapses with correct targets. How is the assembly of neural circuits for dimorphic behaviors molecularly specified and to what extent do sexual differentiation factors interact with these processes? Additionally, many sexually dimorphic behaviors have evolved under sexual selection and are hence rapidly evolving, displaying extensive variation within and between species. With a deep understanding of how dimorphic behaviors develop, we are now in position to explore the molecular processes that underlie their evolution. The groundswell of developmental and neurogenetic tools in recent years puts these challenges within reach.

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