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Molecular mechanisms underlying sexual differentiation of the nervous system

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Abstract

A long-standing goal in developmental neuroscience is to understand the mechanisms by which steroid sex hormones pattern the mammalian central nervous system along male or female pathways to enable subsequent displays of sexually dimorphic behaviors. In this article, we review recent advances in understanding the epigenetic and transcriptional mechanisms mediating sexual differentiation of the brain in mammals, flies, and worms. These studies suggest a model of sexual differentiation wherein master regulators of sex determination initiate a cascade of sexually dimorphic gene expression that controls development of neural pathways and behavioral displays in a strikingly modular manner. With these advances in molecular genetics, it is now feasible to disassemble different components of sexually dimorphic social behaviors without disrupting other behavioral interactions. Such experimental tractability promises rapid advances in this exciting field.

Introduction

Sexually reproducing species exhibit sex differences in social interactions, presumably to enhance reproductive success and survival of progeny. Many of these differences are acquired traits that depend on experience. Nevertheless, a core set of sex-typical behavioral displays such as mating, territorial aggression, and parental care, although modifiable by experience, are innate in the sense that they can be displayed without prior training. These sexual dimorphisms in innate behavior reflect the action of a sexually differentiated nervous system [1–3]. While the chromosomal and endocrine mechanisms mediating sexual differentiation can vary across taxa, such mechanisms converge to regulate sexually dimorphic gene expression that in turn guides sex-typical developmental trajectories and adult function of neural pathways [4]. The molecular mechanisms underlying these sexually dimorphic gene expression programs in the central nervous system remain poorly understood. Because sexual differentiation in mammals depends on the long-lasting actions of early life exposure to steroid hormones, it is likely that epigenetic mechanisms play an

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important role [5,6]. In this review, we highlight recent advances in understanding the transcriptional and epigenetic mechanisms of sexual differentiation of the central nervous system. We illustrate these advances with examples from flies, worms, and mice, and we discuss how modern molecular and genetic approaches should enable unprecedented insights into sexual differentiation of the nervous system.

Development and function of sex-specific circuits in *C. elegans*

The nematode worm *Caenorhabditis elegans* (*C. elegans*) is unique in allowing investigation of sexual differentiation at the level of individual identified neurons, as its nervous system is numerically simpler and mapped in great detail [7,8], with 294 neurons shared between both sexes, 8 neurons that are hermaphrodite-specific, and 91 neurons that are male-specific. These sex-divergent neurons enable molecular dissection of sex-specific neural pathways that mediate specific adaptive behavioral programs in this organism. Hermaphrodites have two X chromosomes whereas males have one (XO), and the number of X chromosomes (or X-to-autosome ratio) determines the sex of the worm [9]. Sexual differentiation occurs in a largely cell-autonomous manner under control of the zinc-finger transcription factor TRA-1 [10–12]. In hermaphroditic cells, TRA-1 represses male-specific genes. In the male, TRA-1 is degraded by proteolysis, allowing development of the male phenotype [13,14]. Importantly, as a result of this system sexually mosaic animals can be generated by regulating TRA-1 expression in specific cells [15]. This allows the generation of ‘male’ neurons in hermaphrodites, or vice-versa, allowing elegant dissection of sex-specific circuits.

Remarkably, in addition to sex-specific neurons, male and hermaphrodite *C. elegans* also possess shared neurons that nevertheless form sex-specific projections and synapses. Recent studies by Oliver Hobert’s group have made progress in describing the cellular and transcriptional mechanisms that mediate the development of sex-specific connections of shared neurons [16••]. The shared PHB sensory neuron forms synapses on to three downstream interneurons in hermaphrodites, while in males it forms synapses on to a different shared interneuron as well as male-specific motor, sensory and inter- neurons. These sexually dimorphic connections appear to arise by at least two distinct mechanisms. PHB efferent synapses develop in both males and hermaphrodites at early larval stages but connections to different shared interneurons are selectively lost in the two sexes (‘selective pruning’). In some other neurons, connections are formed only in larval males and these persist in adult males, providing evidence for a ‘pre-patterning’ mechanism for circuit wiring. PHB neurons mediate sex-specific functions: disruption of the PHB neuron altered locomotion and the behavioral response to noxious chemicals in hermaphrodites, whereas it reduced the ability of males to mate. Sex-specific synapse pruning of PHB connections is cell-autonomous since masculinization or feminization of this neuron elicited pruning of the connections appropriate to the sex of the PHB neuron and not to the sex of the post-synaptic cells. Remarkably, the authors found that the sex of the post-synaptic neurons was also sufficient to lead to rewiring of PHB connectivity such that it was now appropriate to the sex of the post-synaptic neuron. In this latter instance, the authors showed that such rewiring was dependent on the expression of DMD-5 and DMD-11, members of the DM family of transcription factors that govern sex determination or differentiation across metazoans

[4,16••]. It will be interesting to understand the mechanisms whereby such selective sexual differentiation of either a sensory neuron or its post-synaptic partner leads to re-wiring of the pathway.

Work by Hobert's group on the sex-shared PHC neurons provides new molecular insights into repurposing of shared neurons [17••]. PHC neurons appear to be typical sensory neurons in hermaphrodites, with connections to a limited number of shared neurons, that are required for response to harsh touch. By contrast, PHC neurons resemble hub neurons in males, with extensive connectivity with male-specific neurons and to different shared neurons, and they are required for specific aspects of the male mating ritual. This sex-specific connectivity is controlled to a large extent cell-autonomously because altering sexual identity of PHC by manipulating expression of TRA-1, the master regulator of sexual differentiation in worms, rewires connectivity of this neuron accordingly. In keeping with the greater connectivity of male PHC neurons, there is a corresponding transcriptional scaling of many components of the synaptic machinery, including the vesicular glutamate transporter EAT-4. Expression of EAT-4 was dependent on shared transcriptional factors as well as sexually dimorphic utilization of *cis*-regulatory elements that repressed expression in hermaphrodites and boosted it in males. Remarkably, the neuritic arbor of as well as transcriptional scaling in male PHC neurons was regulated cell autonomously by DMD-3, yet another member of the DM family of transcriptional regulators. Whether DMD-3 acts directly by binding to the regulatory elements of the *eat-4* locus is unknown.

Elegant studies in *C. elegans* point the way to a systems-level understanding of how internal states such as hunger and age interact with shared circuitry in a sex-specific manner to enable presumptively adaptive male or hermaphrodite-specific responses [18••]. Adult hermaphrodites show a stronger attraction toward the compound diacetyl (a food cue) than do males. This attraction is mediated by two pairs of sex-shared neurons (AWA and AWC) in hermaphrodites but by AWC in males. This is associated with sex-specific expression of the odorant receptor ODR-10 in hermaphrodite AWA neurons. This sex difference only exists in well-fed adults. Larval AWA neurons of both sexes express this chemoreceptor, and both males and hermaphrodites show equivalent attraction to diacetyl. Food deprivation upregulates *odr-10* in male AWA neurons and increases food-seeking behavior at the expense of seeking mates. Genetically specified circuits can thus be modulated dynamically by external cues to regulate sex differences in behavior; it will be interesting to identify the signaling cascades that modulate chemoreceptor expression in an age and nutritional status dependent manner. Sex-shared sensory neurons can also guide sexually dimorphic responses to pheromones in worms. Males but not hermaphrodites are attracted to ascarosides, components of the worm cuticle that can act as pheromones [19]. This attraction is mediated by a pair of sex-shared sensory neurons (ADF), and feminization of these neurons results in males being repelled by rather than attracted to ascarosides [20•]. The ability of ADF to detect and mediate attraction to ascarosides was dependent on the transcriptional regulator MAB-3, a founding member of the DM family of transcription factors. How MAB-3 regulates ADF function is presently unknown.

Together, these pioneering studies in worms show how sexually dimorphic wiring and behaviors can emerge from neurons present in both sexes. Moreover, they reveal a profound

sexually dimorphic reconfiguration of morphology and function of shared sensory neurons (PHA, PHB, ADF, AWA). Whether such sexually dimorphic reconfiguration is present in other worm sensory neurons, and more generally, is common to sensory neurons in flies or mammals is an open question.

Sexual differentiation in *Drosophila*: the search for isoform- and sex-specific targets of master transcriptional regulators

In the fruit fly *Drosophila melanogaster*, sexual determination is chromosomal as in the worm, such that the number of X chromosomes (or the ratio of X chromosomes to autosomes) determines the sex of the animal [9,21,22]. Sexual differentiation of the nervous system is mediated by two transcription factors, Fruitless (Fru) and Doublesex (Dsx) both of which exhibit sex-specific splice variants in the nervous system (Fru^{M/F}; Dsx^{M/F}) [23]. Strikingly, Fru^M is required for male *Drosophila* to exhibit courtship behavior, and ectopic expression of Fru^M in female neurons is sufficient to induce most components of male courtship behaviors [24–26]. The molecular mechanisms whereby Fru drives sexual differentiation are now beginning to be elucidated. Fru^M exhibits three splice variants (Fru^{MA,MB,MC}), all of which are expressed in the male nervous system. Fru is a BTB-domain-containing zinc finger transcription factor, and each of the A, B and C isoforms contains an alternative DNA-binding domain. Mutations restricted to specific splice isoforms of Fru^M demonstrated separable functions in the development and execution of male courtship behavior [25,27••]. Isoforms B and C are widely expressed in overlapping populations whereas isoform A is expressed in a more restricted set of neurons. Males mutant for isoforms B and C recapitulated some of the behavioral deficits shown by males mutant for all Fru^M isoforms, including male-male courtship, whereas no behavioral phenotypes were discernible in males mutant for isoform A. In addition, there were differences between the phenotypes of males mutant for isoforms B and C, particularly in courtship song production. Together, the findings from this study are consistent with the notion that these isoforms of Fru^M perform distinct as well as redundant functions. Indeed, Goodwin's group demonstrated overlapping and distinct genomic binding sites for these isoforms of Fru^M [27••]. While all three isoforms regulated genes related to the development of the nervous system and regulated many targets in common, they showed differences in consensus motif binding and some non-overlapping targets. This implies some degree of functional differentiation of Fru^M isoforms. Of the 19 potential target genes tested for sexually dimorphic expression, 14 showed higher expression in males, and upon testing, 2 showed male courtship defects following RNAi-mediated knockdown. These findings represent a significant advance in our understanding of how Fru^M regulates various components of the courtship ritual. They suggest a model in which Fru^M, acting via different isoforms, regulates sexually dimorphic expression of target genes that, individually, regulate different aspects of male courtship in a modular manner. This model is supported by a recent study from Yamamoto's group in which they showed that the B isoform of Fru^M represses *robo1* in a specific cluster of neurons through a *cis*-regulatory sequence located in the promoter of *robo1* [28•]. This results in a regulatory switch that guides these neurons down a male or female developmental pathway. This modular mode of Fru^M action is similar to that proposed for mice in which sex hormones drive sexually dimorphic expression of target

genes that in turn regulate different components of male or female social behaviors in a modular manner [29•]. Fru^M can also exert diverse effects through differential coupling with other transcriptional regulators. Another study by Yamamoto's group showed that the effects of Fru^M on development of sex-specific neuronal morphology depended partially on its interaction with two chromatin regulators, HDAC1 and HP1a, that function antagonistically in driving male-typical differentiation of specific groups of neurons [30•]. It remains to be determined whether this interaction with HDAC1 or HP1a is exclusive to a particular Fru^M isoform.

Dsx, which is the other founding member of the DM family of transcription factors (Mab3 in worms being the other), is required for sexual differentiation of peripheral tissues as well as a subset of neurons. While to date no genome-wide studies appear to have identified its targets in the fly brain, a genome-wide study of Dsx^M and Dsx^F targets in gonad and fat tissue by Oliver's group may be instructive in understanding the sex-specificity of this transcription factor [31••]. They found that the vast majority of Dsx targets were bound by both male and female isoforms in multiple tissues. Furthermore, only a minority of genes with Dsx association in their genomic regions showed expression changes upon *dsx* knockdown or upon switching isoforms. However, many of the most highly-occupied Dsx target genes showed sex-specific defects upon knockdown. These were sometimes strikingly tissue- and sex-specific; for example, knockdown of the Dsx target *Iola* caused almost total loss of external genitalia in females and deformation of the testes in males. Thus, shared targets of Dsx mediate distinct developmental programs in the two sexes. This work is intriguing in that it suggests that although males and females express different isoforms of Dsx that drive different developmental programs, their sex-specific effects are not apparent from their occupancy in chromatin but presumably depend on downstream signaling events or differential interaction with other transcriptional regulators.

The requirement for both Dsx and Fru^M in masculinization of the fly nervous system raises the question of whether these transcription factors co-regulate target gene expression in neurons. Recent work by Baker's group suggests that at least for Lgr3, a member of the relaxin receptor family of GPCRS, the answer is more complicated [32•]. They showed that the B isoform of Fru^M inhibits Lgr3 expression in median bundle neurons via an intronic *cis*-regulatory element whereas Dsx^F inhibits and activates Lgr3 expression via two distinct *cis*-regulatory elements in two different pools of abdominal ganglionic neurons. These findings demonstrate unexpected complexity in the regulation of sexually dimorphic expression of a single gene in different neuronal populations.

These recent studies have advanced our understanding of how master regulators drive sexual differentiation of the developing *Drosophila* nervous system. However, refinements to sex-specific circuits can also occur post-eclosion. Wang's group elegantly elucidated mechanisms underlying the ability of older male flies to outcompete younger males in copulating with females [33••]. They showed that the success of older males was dependent on increased sensitivity of Or47b-expressing chemosensory neurons to the courtship-promoting cuticular pheromone palmitoleic acid. In turn, this increase in sensitivity of Or47b neurons is mediated by juvenile hormone acting via its cognate receptor Met in Or47b neurons. This later differentiation event underscores how the sexually dimorphic

nervous system is fine-tuned to maximize the display of behaviors that promote reproductive success. This study is also interesting as it highlights a case in which the maturation of a circuit important for social behaviors in flies is *not* cell-autonomous but requires hormonal signaling, as is the case in mammals.

Sexual differentiation of the mouse brain: emerging evidence for a role of epigenetic modifiers

While sexual differentiation in worms and flies is largely cell-autonomous, in most mammals it proceeds by both chromosomal and endocrine mechanisms [1–3]. The Y-linked gene *Sry* is necessary and sufficient for differentiating the bipotential gonad in midgestation mouse embryos into testes. In the absence of *Sry*, the bipotential gonad is pre-patterned to differentiate into ovaries. During a critical perinatal window (the exact time can vary depending on the species) in mice, there is a transient surge of testosterone that is thought to irreversibly masculinize a bipotential brain (organizational action of sex hormones). Many of the actions of testosterone on the brain appear to be mediated by its estrogenic metabolites, with 17 β -estradiol being the most biologically potent estrogen *in vivo*. Such conversion of testosterone into estrogens is catalyzed by the enzyme aromatase expressed in discrete regions of the mouse brain [34]. By contrast, the ovaries are quiescent at this stage, and therefore the male brain is selectively exposed to testosterone and estrogen during this critical organizational period. Following puberty, there is a rise in sex hormones in the circulation in both sexes, and these hormones modulate physiological functioning (activational action of sex hormones) of developmentally wired circuits to enable the display of sexually dimorphic social behaviors [1–3]. The sex steroids testosterone, estrogens, and progesterone act through specific nuclear receptors that are ligand-activated transcription factors and are also found in chromatin-modifying complexes [35,36]; in addition, they also exert rapid, non-transcriptional (traditionally referred to as “non-genomic”) effects by interacting with various signal transduction pathways (reviewed in [1]). Sex hormone receptors may therefore exert their organizational and activational effects on the brain through diverse mechanisms. Previous work by Xu and colleagues found genes in multiple regions of the mouse hypothalamus and amygdala that 1) showed sexually dimorphic expression, 2) were regulated by circulating steroid hormones, and 3) affected specific components of adult sexually dimorphic social behaviors [29]. At least some of these genes showed evidence of epigenetic programming. A particularly striking example is the gene *Cckar*, which is required for the high wildtype level of female sexual behavior. This neuropeptide receptor is more highly expressed in the female ventromedial hypothalamus, ventrolateral region (VMHvl) than in males. Castration of adult females reduces its expression and eliminates the sex difference. Estrogen treatment of adult females restores its expression, but treatment of castrated adult males with estrogen does not elevate *Cckar* expression to female-typical levels. Thus, sex hormone-regulated genes in the mammalian brain are expressed in a sexually dimorphic manner, likely to be epigenetically regulated, and relevant for the display of social behaviors. The full extent of sex hormone regulated sexually dimorphic gene expression and the molecular mechanisms underlying these sex differences are presently unknown.

The complexity and cellular heterogeneity of the mammalian brain renders the search for epigenetic and transcriptional regulators of sexual dimorphism difficult. Nevertheless, progress has been made in establishing that epigenetic processes are at work in establishing sex differences in the developing and adult mammalian brain. Several groups have looked at histone modifications as candidate mechanisms for sexual differentiation. These studies find changes in methylation or acetylation at key lysine residues and show that experimental alteration of these modifications can alter sexual differentiation of cell number in particular brain regions or modify adult social behaviors [37•,38•,39•,40•].

Another candidate mechanism for epigenetic programming of sexually dimorphic gene expression is DNA methylation, which is typically associated with gene repression. Recent work by Forger's group showed that pharmacological inhibition of DNA methyltransferase activity at birth feminized expression of *Esr1*, a nuclear hormone receptor for estrogen, in two hypothalamic nuclei [41•]. This suggests that DNA methylation may establish or maintain male-specific gene repression in these regions. Interestingly, the McCarthy lab found that neonatal female rats had higher DNA methyltransferase activity in the preoptic area (POA). Whereas Forger's group found that inhibition of DNA methylation feminized specific gene expression patterns in male mice, McCarthy's group found that such inhibition masculinized neural spine morphology in female rats [42•]. Thus, epigenetic regulation of sexual differentiation can modify different sexual differentiation events in a sex-specific manner. Regardless, such developmental effects of methylation not only have acute effects on sexual differentiation but also result in longer lasting effects that persist into adulthood, consistent with the notion that epigenetic processes have enduring effects [42•,43•].

Future directions

We are excited by the recent advances in unraveling the molecular mechanisms that drive sexual differentiation of the nervous system. Although the mechanisms that regulate sexual differentiation appear different between worms, flies, and mice, there are also commonalities. As we have discussed, the DM family of transcription factors plays an important role in sexual differentiation in both worms and flies; intriguingly, the mouse genome contains several loci that code for DM family related transcription factors (DMRTs) [44]. However, the function of these genes in sexual differentiation in mice remains unclear. Moreover, converging evidence from worms, flies, and mice suggests that such master regulators of sexual differentiation drive sex differences in gene expression that in turn control cellular changes in specific neurons and regulate discrete components of sexually dimorphic behaviors in a modular manner. While the scale of the problem of unraveling the molecular determinants of sexually dimorphic circuit development is greater in mammals than in worms or flies, recent work suggests that it should be feasible to not only identify sexually dimorphically expressed genes but also to elucidate their function and the function of the underlying neural circuits [1]. Together, the combined use of cell type targeted genome-wide studies and the powerful toolkit for manipulating neural circuits [45] should lead to exciting and novel insights into how an apparently similar brain generates distinct behavioral repertoires in the two sexes.

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Highlights

- Master regulators of sex determination initiate program of sexually dimorphic gene expression that controls sexual differentiation of neural circuits and behavior in a modular manner
- In worms, transcriptional and genomic mechanisms that regulate sexually dimorphic gene expression are beginning to emerge
- In flies, sex-specific isoforms of Fru and Dsx regulate shared and sexually dimorphic gene expression patterns and neuronal morphology
- In mice, molecular mechanisms that mediate sexually dimorphic gene expression and modulate behavior are being identified