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Control of masculinization of the brain and behavior

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Sex steroid hormones exert a profound influence on the sexual differentiation and function of the neural circuits that mediate dimorphic behaviors. Both estrogen and testosterone are essential for male typical behaviors in many species. Recent studies with genetically modified mice provide important new insights into the logic whereby these two hormones coordinate the display of sexually dimorphic behaviors: estrogen sets up the masculine repertoire of sexual and territorial behaviors and testosterone controls the extent of these male displays.

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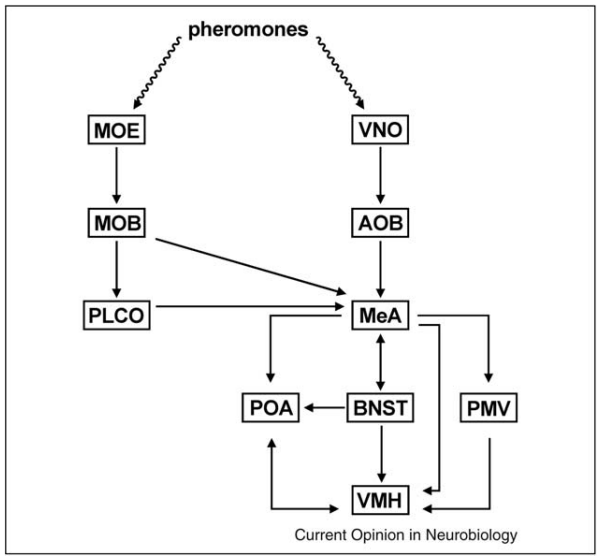
Introduction

All sexually reproducing animals display gender differences in behavior that are characteristic of the species. These sexual dimorphisms are most obvious in behaviors related to reproduction such as courtship displays, territorial aggression, mating, and parental care. Such sex differences in behavior occur because the nervous system differs between males and females. The display of sex typical behaviors can be observed in animals without prior experience or training, indicating that the underlying neural circuits are developmentally programmed to be dimorphic [1]. This review focuses on recent advances in our understanding of the mechanisms underlying sexual differentiation of the brain and behavior in mice. There are many similarities as well as important differences in the dimorphic development of the brain and behavior in other species; although these are beyond the scope of our discussion, they have been reviewed extensively elsewhere [2–12].

In mice and many other mammals, sexual differentiation of the brain proceeds largely under the control of steroid hormones produced by the gonads. Gonadal differentiation is directed by genes on the sex chromosomes such that *Sry*, a gene on the male specific Y chromosome, is determinative for the testes [13]. The absence of Sry in females allows the gonadal primordia to differentiate into the ovaries. The development and function of the neural pathways that mediate sexually dimorphic behaviors are tightly regulated by gonadal steroid hormones. The developmental or 'organizational' effect of sex steroids results in long-lasting changes in the nervous system and behavioral displays, whereas the subsequent 'activational' effect of these hormones in sexually mature animals leads to transient alterations in neural function and behavior [14•,15]. While these two circumscribed effects of sex steroids have served a useful heuristic function, not all sexually dimorphic behaviors differentiate under a sex specific organizational and activational role of gonadal hormones. For example, both male and female mice exhibit male pattern sexual behavior toward females, although females will display such behaviors at lower frequency [16,17••,18••]. The frequency of male pattern mating is directly regulated by adult levels of testosterone such that wildtype females supplemented with testosterone mount females as readily as males [19]. Gonadal steroids also control neuronal differentiation in peripubertal animals, a time period that straddles the traditional perinatal organizational window and the adult activational phase [20]. In any event, previous research has demonstrated that gonadal hormones regulate sexual differentiation of neuronal number, connectivity, and gene expression within limbic brain regions that influence mating, aggression, territorial marking, and maternal care (Figure 1) [1,18••,21]. These differentiation events provide the cellular correlates, and presumably the neural substrates of gender dimorphisms in behavior, of the transient and enduring effects of sex hormones.

Classic studies in many vertebrates, including mice, show that male patterning of the brain and behavior requires estrogen as well as testosterone signaling via their cognate receptors [1,22]. This is surprising because estrogen is essentially undetectable in the male circulation. However, testosterone, or a related androgen, is an obligate precursor of estrogen *in vivo*, and circulating testosterone in males acts as a source of estrogen in the brain. Thus, testosterone appears to serve a dual role: it activates its receptor, the androgen receptor (AR), in neural circuits that control male behaviors, and it also serves as a precursor for estrogen, which in turn influences neural circuits via the estrogen receptors. However, the dual

Figure 1



Neural pathways underlying sexually dimorphic behaviors. Many hypothalamic and amygdalar centers have been implicated in the control of sex specific behaviors. Each of these brain regions is sexually dimorphic and expresses one or more gonadal hormone receptors [37,38]; in addition, the BNST, MeA, POA, and VMH also express aromatase [18••], and represent sites of estrogen synthesis in the adult brain. Some of these nuclei and their connections, including with pheromone sensing neurons in the MOE and VNO, are illustrated in this schematic [31,32,54,70,71]. AOB, accessory olfactory bulb; BNST, bed nucleus of the stria terminalis; MeA, medial amygdala; MOB, main olfactory bulb; PLCO, posterolateral cortical amygdala; PMV, ventral premamillary nucleus; POA, preoptic hypothalamus; VMH, ventromedial nucleus of the hypothalamus.

requirement of testosterone and estrogen in male behaviors can also be explained by a more parsimonious model in which testosterone is limited to acting as a prohormone for estrogen in the brain. In this case, the entire range of male type patterning of the brain and behavior is engendered by estrogen signaling. Recent studies provide new insight into this outstanding issue, and these developments are discussed below. What are the molecular effectors of sex steroid signaling that drive dimorphic behaviors? While these remain to be identified for the most part, a recent study has identified many genes that appear imprinted in a sex specific manner; the implications of these exciting findings on sexual differentiation of the brain and behavior are also discussed.

The role of estrogen in controlling dimorphic behaviors

Estrogen signaling is critical for male behaviors. The loss of masculine behaviors that follow adult castration can largely be rescued by the provision of estrogen [23]. Moreover, male mice null for the two nuclear estrogen receptors, ERα and ERβ, do not mate or fight [24]. Testosterone is metabolized into estrogen via the converting enzyme aromatase, which is expressed in the brain, and males null for aromatase also exhibit deficits in mating and fighting [25–28]. How does estrogen control dimorphic behaviors in both sexes? Circulating estrogen from the mother is sequestered by alpha fetoprotein in the fetal circulation, thereby precluding access of the hormone to the brain [29]. On the day of birth, there is a male specific surge of circulating testosterone that is converted to estrogen in the brain [30]. By contrast, the ovaries are quiescent in female pups, and the brain is not exposed to sex steroids in this time period. Thus, only the neonatal male brain is exposed to testosterone and estrogen; as is the case with the ovaries, differentiation of the female brain, at least in the neonatal period, may follow a default program that is preset by patterning genes and is independent of sex hormones.

It has been difficult to visualize the sites of estrogen synthesis in the brain because aromatase is expressed at low levels, precluding detection at cellular resolution using traditional approaches. This problem has been addressed recently by genetically tagging the aromatase locus with the reporter genes *nuclear LacZ* and *placental alkaline phosphatase* (*PLAP*), whose products label the nucleus and processes of cells expressing aromatase, respectively [18••]. Aromatase is expressed in a surprisingly sparse manner in the adult mouse brain, and the genetic reporters reveal sexual dimorphisms in the cell number and connectivity of aromatase expressing neurons. There are more aromatase positive neurons in the medial amygdala (MeA) and the principal nucleus of the bed nucleus of the stria terminalis (BNST) in males compared to females. The anterior hypothalamus and the ventromedial nucleus (VMH) contain significantly more innervation from aromatase expressing neurons in males compared to females. This sensitive unbiased genetic approach also reveals a small pool of neurons expressing aromatase in the female caudal hypothalamus that is essentially absent in the male brain. The MeA, BNST, and VMH have previously been implicated in the control of reproductive and territorial behaviors (Figure 1) [31,32]. Of particular note, ERα in the VMH controls female sexual behavior [33], whereas electrical stimulation of the male VMH elicits attack behavior [34]. One possible role for the dimorphic innervation of the VMH therefore may be to regulate these sex specific behavioral outcomes.

The masculinization of aromatase expressing neurons is regulated by estrogen signaling in the neonatal period and is independent of AR function [18••]. Estrogen supports sexual differentiation of aromatase expression at least in part by promoting neuronal survival in the MeA and BNST in the male brain [18••,35]. Estrogen supplementation to neonatal females is sufficient to masculinize aromatase expression, and the male typical patterns of number and connectivity of aromatase expressing

neurons persist without additional manipulations. As adults, these females exhibit a dissociation in sex typical behaviors: they are not receptive to males, they mate with females at the low frequency typical of wildtype females, but they mark territory and attack males similar to wildtype males. These behavioral findings are in agreement with previous hormone supplementation studies that demonstrated the defeminizing effects of early estrogen exposure on sexual behavior [36]. However, the new findings further demonstrate that the adult ovaries of females treated with estrogen neonatally support male typical territorial fighting and urine marking. In other words, the adult gonads of both sexes can support masculine territorial behaviors provided the animal has been exposed to estrogen neonatally.

The role of testosterone in controlling dimorphic behaviors

Several lines of investigation implicate testosterone signaling via AR in the control of male pattern behaviors. AR is expressed in each of the neuronal pools that has been shown to control dimorphic behaviors, including the MeA, BNST, preoptic hypothalamus (POA), and VMH (Figure 1) [37,38]. Its expression is also sexually dimorphic such that there are more AR positive neurons in the male BNST and POA compared to these centers in the female [37]. Estrogen administered to adult castrate wildtype males does not restore mating and fighting to levels observed in intact males [39,40]. Full rescue of male behavior in castrates can be effected with testosterone or provision of dihydrotestosterone, a non-aromatizable androgen that activates AR, along with estrogen. Finally, male mice mutant for AR do not mate or fight, indicating a prominent role for testosterone signaling via its receptor to drive male behaviors [41,42]. One caveat of this interpretation is that a constitutive deletion of AR leads to postnatal testicular atrophy and minimal testosterone in the circulation. Consequently, the behavioral deficits in these mutant males may result solely because of inadequate estrogen synthesis and signaling in the brain.

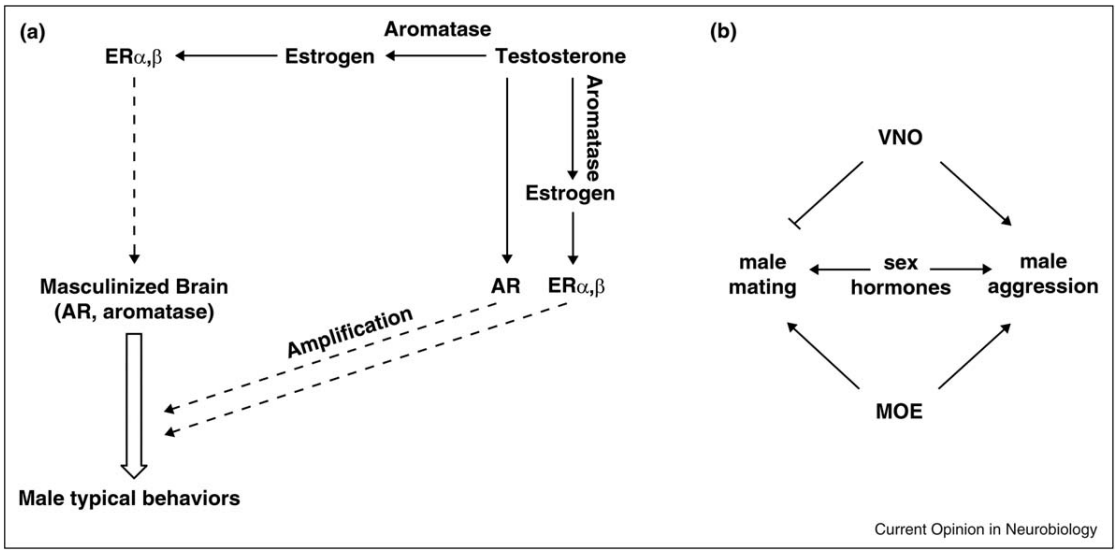
Two groups have recently used a Cre-loxP strategy to delete AR in the nervous system, thereby bypassing the peripheral requirements for AR function [43••,44••]. Mutant males with a deletion of AR in the nervous system have intact testes and circulating testosterone, thereby permitting an analysis of the role of AR in the neural pathways that control male behaviors [43••]. These mutant males display a masculinized repertoire of mating and territorial fighting and marking. However, the mice display striking deficits in the extent or frequency of male behaviors. Although all males will eventually mate with females and sire litters, they are less likely to mate in short term assays, thereby indicating a lowered probability of initiating sexual behavior. These mutants fight less intensely with intruder males, and they mark their territory with fewer urine marks compared to wildtype males. Taken together, these results provide strong evidence for AR in regulating the extent, but not the programming, of male behaviors. In accord with this notion, there is minimal AR expression in the neonatal male brain at the time of the testosterone surge. Thus, the neonatal developmental events that masculinize the brain occur at a time when there is ample testosterone in the circulation, conversion of this hormone into estrogen in the brain, but little AR expression in the relevant neural circuits. By contrast, aromatase and the estrogen receptors are expressed at high levels in the limbic brain at the time of birth, and they are well poised to regulate sexual differentiation of the brain. Indeed, estrogen signaling is necessary and sufficient for the male typical pattern of AR expression in the brain [43••].

Constitutive AR mutants fail to mate or fight whereas mice with a restricted deletion of AR in the nervous system display male typical sexual and aggressive behaviors, albeit with deficits in the frequency or extent of these displays [42,43••,44••]. The distinct phenotypes likely reflect the differences in circulating testosterone and estrogen synthesis in the brain: constitutive mutants have little circulating testosterone whereas mice with AR deletion in the nervous system have testosterone titers within the normal range. Indeed, supplementing adult males bearing a constitutive AR loss-of-function allele with testosterone or estrogen elicits masculine patterns of sexual and aggressive displays at levels that resemble those observed in mice with a deletion of AR in the brain [18••,42,43••,45,46]. How might AR regulate the extent of male behavioral displays? An elegant solution is suggested by the finding that testosterone signaling through AR controls the levels of aromatase activity in the brain [47]. However, provision of estrogen to constitutive AR mutants does not restore male behaviors to wildtype levels [45], suggesting additional roles for this receptor in regulating male typical behaviors.

A working model for the control of sexually dimorphic behaviors

In conjunction with previous work, the recent findings [18••,43••,44••] suggest a surprisingly simple model for the hormonal control of male sexual behavior and territoriality (Figure 2a): estrogen masculinizes the neural circuits for mating, aggression, and urine marking, and it is sufficient to elicit baseline levels of male patterns of these behaviors in the adult animal. Testosterone, signaling via AR, serves to amplify the display of these masculine behaviors to levels observed in the intact adult male. The current findings do not exclude a redundant role for testosterone signaling via AR in the sexual differentiation of the male brain and behavior. Nevertheless, male mice mutant for both nuclear estrogen receptors show a complete loss of masculine behaviors despite apparently normal levels of circulating testosterone

Figure 2



Model for the control of male typical sexual and territorial behaviors. **(a)** Schematic representation illustrating that masculinization of the neural substrates for mating and territorial behaviors, including the expression of aromatase and AR [18••,43••], proceeds largely under the control of estrogen, which is synthesized in the brain from circulating testosterone via the action of aromatase. Both estrogen and testosterone, signaling via their cognate receptors, act on this masculinized substrate to activate male pattern behaviors. **(b)** Genetic inactivation of odor evoked activity in the MOE or VNO shows that both chemosensory pathways control male mating and aggression [52–56]. The MOE and VNO are essential for male aggression in a non-redundant manner. The MOE is essential for male sexual behavior, whereas the VNO appears to inhibit it. MOE signaling in conjunction with the high titer of testosterone increases the frequency of male courtship toward females, whereas females, who have low titers of this hormone, exhibit male type mating toward other females at a reduced frequency. The same repertoire of pheromonal receptors appears to be expressed in the two sexes, indicating that males and females can sense male pheromones, which inhibit male type mating in both sexes and promote aggression in males. This suggests that the central pathways that process pheromonal or hormonal cues for male typical fighting may be sexually dimorphic.

[24,48]. Moreover, estrogen administration to neonatal females is sufficient to masculinize most cellular and molecular dimorphisms, including those in AR and aromatase expression [18••,43••]. As adults, such females exhibit low intensity male pattern behaviors in response to the hormones produced by their ovaries; the levels of these behaviors resemble those observed in wildtype males when the females are supplemented with testosterone to recapitulate the titers of sex hormones in the intact male [18••]. This testosterone-induced amplification in male typical behaviors is probably mediated at least in part by AR because mice null for AR in the brain mate, fight, and mark territory at low levels similar to those displayed by females treated solely with estrogen neonatally [18••,43••].

Although gonadal hormones exert inordinate influence on sexually dimorphic behaviors, these displays are elicited in response to specific sensory cues. In mice and many other animals, behaviors such as mating and aggression are triggered in response to pheromones, which are chemosensory cues that convey reproductive and other social information about an animal to members of the species [49,50]. Pheromones are recognized by neurons in two distinct sensory epithelia in the nose, the main olfactory epithelium (MOE) and the vomeronasal organ (VNO) (Figure 1). Neurons in the MOE and VNO utilize distinct signal transduction mechanisms to detect pheromones, permitting genetically targeted loss-of-function of the MOE or VNO in separate mouse mutants. Targeted loss of odor-evoked activity in sensory neurons of the MOE leads to profound reduction in male sexual behavior and aggression (Figure 2b) [51–54]. Genetic deletion of Trpc2, a cation channel required for olfactory cue evoked signaling in the VNO, also eliminates male fighting, indicating a dual requirement for both the MOE and VNO in recognizing pheromones that elicit aggression (Figure 2b) [55,56]. Studies with Trpc2 mutants further indicate that the VNO inhibits masculine sexual behavior because Trpc2 mutant males and females mate with mice of either sex (Figure 2b) [17••,55,56]. Taken together, these findings suggest that activation of MOE sensory neurons with pheromones emitted by either males or females may disinhibit the display of male sexual behavior. Male pheromones may normally inhibit sexual behavior and promote aggression between males; such aggression eliciting cues may convey a 'do not mate' or an 'attack' signal. The repertoire of chemoreceptors does not appear to be sexually dimorphic, suggesting that females also recognize such male pheromones; they

may not attack males, however, because of a sexually dimorphic processing of these cues. Wildtype males and females mate with other females, although males do so with higher probability. This mating frequency is likely regulated by testosterone since females provided with testosterone display male sexual behavior at levels indistinguishable from normal males [19]. Consistent with this notion, male mice mutant for AR in the nervous system mate with females with a low probability similar to wildtype females [43••]. Thus, the low frequency of male sexual behavior normally exhibited by wildtype females toward other females may be a consequence of two non-redundant mechanisms: inhibitory input from the VNO and minimal circulating testosterone (Figure 2b).

Recent work has revealed the sex specific nature of some pheromones that are recognized by the VNO or the MOE [57,58••,59••,60,61,62•]. One class of such pheromones, major urinary proteins (MUPs), is present in male but not in female urine, activates VNO but not MOE sensory neurons, and can trigger intermale aggression [58••]. Another peptidergic pheromone, ESP1, is secreted by the male lacrimal glands and increases female sexual receptivity by activating sensory neurons in the VNO [59••]. Several studies have also identified the presence of additional dimorphic chemosensory cues in mouse urine and saliva, whose behavioral relevance will be fascinating to elucidate [60,61,62•].

Most neural functions, including motor output, are shared between the sexes. The presence of sex differences in behavior therefore reflects molecular or structural dimorphisms in neural circuits that alter the response of the animal to external sensory cues or internal regulators such as hormones. Both chemosensory cues and hormonal signals coordinate sexually dimorphic behaviors. Where and how these two control systems converge to regulate these behaviors remains to be determined. However, each of the dimorphic, sex steroid receptor expressing regions we have discussed, including the MeA, BNST, POA, and VMH, is an integral component of a multi-synaptic olfactory pathway, suggesting that the sensory and hormonal control of these social behaviors is regulated at many nodes in the underlying neural circuits (Figure 1).

The sex steroid receptors we have discussed are ligand-activated transcription factors that can directly modulate expression of target genes. While the expression of several genes is regulated by gonadal hormones, it remains to be demonstrated whether these genes represent direct transcriptional targets of steroid hormone receptors. Several important studies indicate the existence of hormone-independent, sex chromosome-based genetic loci that mediate sexual differentiation of the brain [14•,63–65]. Some of these studies utilize a genetic cross in which the Y chromosome carries a spontaneous deletion of *Sry*, the sex determining locus, and an autosome bears a translocated *Sry*. This clever strategy dissociates the presence of testes from the presence of the Y chromosome, thereby permitting an analysis of the effects of the sex chromosomes on sexual differentiation of the brain independent of testicular hormones. One caveat of these studies is that the genetic cross does not yield wildtype male mice (male mice with an intact Y chromosome) for a direct comparison with the experimental genotypes obtained from this cross. Nevertheless, these studies reveal the control of some aspects of sexual differentiation of the brain and behavior in a hormone-independent but sex chromosome-dependent manner. It will be extremely interesting to identify the genetic loci responsible for these phenotypes. More recently, a large set of imprinted genes, many of which are imprinted in a sex specific manner, has been identified in the mouse brain [66••,67••]. Unlike most previously identified imprinted loci, the vast majority of the newly identified genes do not exhibit an absolute bias of maternal or paternal allelic expression. Rather, there is a parent-of-origin bias in expression of the two alleles, and this bias is sexually dimorphic for many loci. Previously identified imprinted genes in the brain have been shown to regulate maternal behaviors as well as other social interactions, suggesting that the newly identified loci may also regulate social behaviors. The molecular mechanisms that guide sex specific imprinting are unknown, and it is possible that novel, perhaps sex hormone based, regulatory mechanisms are involved.

Future studies

There has been tremendous progress in the past few years in understanding the sensory and hormonal control of sexual and territorial behaviors. It will be interesting to determine whether sensory and hormonal cues control other sex differences in behavior such as anxiety in a similar manner [68]. Recent large scale sequencing efforts have suggested new modes of sexual differentiation of the brain, and one anticipates that future studies will also identify the molecular targets of hormone signaling in the brain. Newly developed tools for neural circuit mapping and manipulation will provide insight into how sensory input is integrated with internal regulators such as hormones to produce sex differences in social behaviors [69]. The mechanisms underlying sex determination and sexual differentiation of the brain and behavior evolve rapidly during speciation. The recent advances in genetic manipulation of non-classical model organisms will permit comparative analyses of these differences at the molecular and circuit level and should provide novel insights into the processes that drive the evolution of these behaviors.

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This study shows that there is minimal AR expression in the brain at the time of the neonatal testosterone surge and that sexual differentiation of AR expression is controlled by estrogen. This suggests that AR is unlikely to organize the circuits for male sexual and territorial behaviors. Indeed, these authors find that male mice with a targeted deletion of AR in the nervous system exhibit a masculinized repertoire of mating, aggression, and marking behaviors, albeit with striking deficits in the levels at which these behaviors are displayed.

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