REVIEW

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Sexual differentiation of the vertebrate nervous system

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Understanding the mechanisms that give rise to sex differences in the behavior of nonhuman animals may contribute to the understanding of sex differences in humans. In vertebrate model systems, a single factor—the steroid hormone testosterone—accounts for most, and perhaps all, of the known sex differences in neural structure and behavior. Here we review some of the events triggered by testosterone that masculinize the developing and adult nervous system, promote male behaviors and suppress female behaviors. Testosterone often sculpts the developing nervous system by inhibiting or exacerbating cell death and/or by modulating the formation and elimination of synapses. Experience, too, can interact with testosterone to enhance or diminish its effects on the central nervous system. However, more work is needed to uncover the particular cells and specific genes on which testosterone acts to initiate these events.

The steps leading to masculinization of the body are remarkably consistent across mammals: the paternally contributed Y chromosome contains the sex-determining region of the Y (*Sry*) gene, which induces the undifferentiated gonads to form as testes (rather than ovaries). The testes then secrete hormones to masculinize the rest of the body. Two of these masculinizing testicular hormones are antimullerian hormone, a protein that suppresses female reproductive tract development, and testosterone, a steroid that promotes development of the male reproductive tract and masculine external genitalia. In masculinizing the body, testosterone first binds to the androgen receptor protein, and then this steroid-receptor complex binds to DNA, where it modulates gene expression and promotes differentiation as a male. If the *Sry* gene is absent (as in females, who receive an X chromosome from the father), the gonad develops as an ovary, and the body, unexposed to testicular hormones, forms a feminine configuration. The genitalia will only respond to testicular hormones during a particular time in development, which constitutes a sensitive period for hormone action: hormonal treatment of females in adulthood has negligible effects on genital morphology[1].

Of the two gonadal hormones that masculinize the body, it is testosterone that also masculinizes the brain. Scientists first demonstrated this by exposing female guinea pigs to testosterone *in utero*, which permanently interfered with the animals' tendency to show female reproductive behaviors in adulthood[2]. Treating adult females with testosterone had a transient effect, or none at all, on these behaviors. Early exposure to steroids such as testosterone also masculinizes brain structures. In this review, we will contrast the various mechanisms by which testosterone masculinizes the central nervous system, discuss the unknowns that remain and relate these findings to human behavior.

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Apoptosis and sexual dimorphism in the nervous system

Lesions of the entire preoptic area (POA) in the anterior hypothalamus eliminate virtually all male copulatory behaviors[3], whereas lesions restricted to the sexually dimorphic nucleus of the POA (SDN-POA) have more modest effects, slowing acquisition of copulatory behaviors[4]. The volume of the SDN-POA in rats is several-fold larger in males than in females (**Fig. 1**). Treating female rats with testosterone just before and just after birth causes the SDN-POA in adulthood to be as large as in normal males[5], whereas castrating male rats at birth results in a smaller, feminine SDN-POA in adulthood. Thus, sexual differentiation of this nucleus resembles that of the genitalia—male hormones early in life permanently masculinize this brain region.

One difference is that it is not testosterone itself, but a metabolite of testosterone, that masculinizes the SDN-POA. The enzyme aromatase, which is abundant in the hypothalamus, converts androgens (such as testosterone) into estrogens (such as estradiol). Estrogen then interacts with estrogen receptors, not androgen receptors, to induce a masculine SDN-POA. Naturally occurring cell death seems to regulate sexual differentiation of the SDN-POA (**Fig. 2**). There are more dying cells in the SDN-POA of neonatal females than males[6], and treating newborn females with testosterone reduces the number of dying cells, which presumably leads to a larger SDN-POA[6]. In contrast, hormone manipulations in adulthood, after the period of naturally occurring neuronal death, have no effect on the volume of this nucleus[7].

The ferret SDN-POA also has a greater volume in males than in females due to perinatal stimulation of estrogen receptors in males, but in this species, cell death does not seem to be involved in sexual differentiation[8]. Unfortunately, Nissl stain does not reveal an SDN-POA in mice[9], but cells containing androgen receptors demarcate a sexually dimorphic area in the mouse POA that resembles the rat SDN-POA[10]. If the areas are homologous, then scientists may be able to use genetic tools available in mice to discern the molecular events involved in masculinization of this nucleus.

Sexually dimorphic brain regions are not always larger in males; the anteroventral periventricular nucleus (AVPV), part of the hypothala-

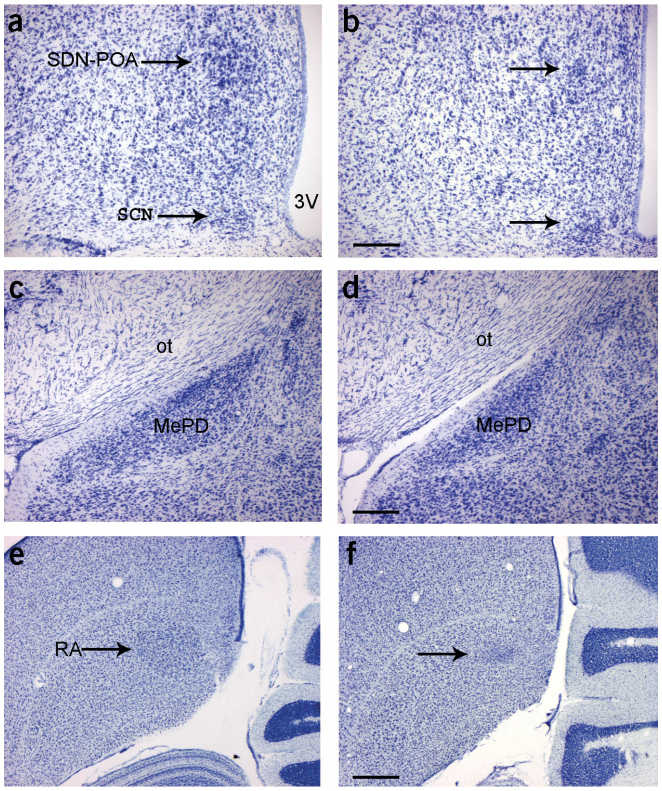

Figure 1 Sexual dimorphisms in the brain. (**a**,**b**) The sexually dimorphic nucleus of the preoptic area (SDN-POA) is larger in male rats (**a**) than in females (**b**) because the testes secrete testosterone during the perinatal sensitive period. After that time, testosterone has little effect on SDN-POA volume. (**c**,**d**) In contrast, the volume of the rat posterodorsal medial amygdala (MePD), which is about 1.5 times larger in males (**c**) than in females (**d**), retains its responsiveness to testosterone throughout life. (**e**,**f**) In zebra finches, the robustus archistriatum (RA) nucleus is crucial for song production and has a greater volume in males (**e**) than in females (**f**). Like the rat SDN-POA, exposure to steroid hormones early in life is essential for the RA to develop a masculine phenotype. For the RA, however, the steroids may not originate from the testes, but are rather synthesized locally in the brain itself. SCN, suprachiasmatic nucleus; 3V, third ventricle; ot, optic tract. All scale bars = 250 μm.

mic system regulating ovulatory cycles, is larger in females than in males, in both mice and rats[11]. As with the SDN-POA, sexual differentiation of the AVPV is due to the actions of aromatized metabolites of testosterone on estrogen receptors, which modulate apoptosis early in life; hormone manipulations in adults have no effect. In contrast to the SDN-POA, masculinization of the AVPV is due to testosterone-induced apoptosis, resulting in a smaller nucleus in adult males than in females[11]. The opposing responses of SDN-POA and AVPV to testosterone indicate that the molecular makeup of the cells targeted by hormone in these two systems must differ.

The spinal nucleus of the bulbocavernosus (SNB) also relies on apoptosis for sexual differentiation. These motor neurons in the lumbar spinal cord innervate striated muscles that attach to the base of the penis[12]. Male rats have more and larger SNB motor neurons than do females, and similar dimorphisms have been found in humans[13] and green anole lizards[14]. Rats of both sexes have SNB motor neurons and their target muscles before birth, but these components die in females around the time of birth unless they are exposed to testosterone[15,16]. The effect of testosterone relies exclusively on androgen receptors. Although adult SNB motor neurons possess androgen receptors, the primary effect of testosterone is to prevent death of the target muscles, which then secondarily spare SNB motor neurons from apoptosis (**Fig. 3**). Thus, testosterone does not act directly on SNB neurons to keep them alive. For example, SNB motor neurons with dysfunctional androgen receptor genes are nevertheless spared from apoptosis by testosterone treatment[17].

Testosterone also acts on AVPV neurons, causing them to release a chemoattractant that establishes a sexually dimorphic innervation pattern[18]. Thus, steroids can induce one cell population to transynaptically masculinize another. Moreover, the same hormone that promotes neuronal apoptosis in the AVPV can prevent it in other systems (SDN-POA, SNB). Mice that overexpress the anti-apoptotic gene *Bcl2* show reduced sex differences in both the SNB and the AVPV[19], suggesting that the *Bcl2* gene mediates sexual differentiation in both structures, but that testosterone modulates expression of this gene in opposite directions to either promote or prevent apoptosis.

Sexual differentiation of the brain in adulthood

Arginine vasopressin fibers innervate the septal region in rats and contribute to pair bonding and parental behavior in a variety of rodents (see accompanying review[20] in this issue). The extent of this innervation is greater in males than in females, but testosterone during development is not sufficient to fully masculinize the region; the hormone must also be present in adulthood[21]. The posterodorsal medial amygdala (MePD), which receives input from olfactory and pheromonal centers and is important for male sexual arousal, depends even more on adult testosterone. MePD volume is about 1.5 times larger in males than in females in rats and mice (**Fig. 1c,d**)[22,23], and testosterone manipulations in adulthood can completely reverse

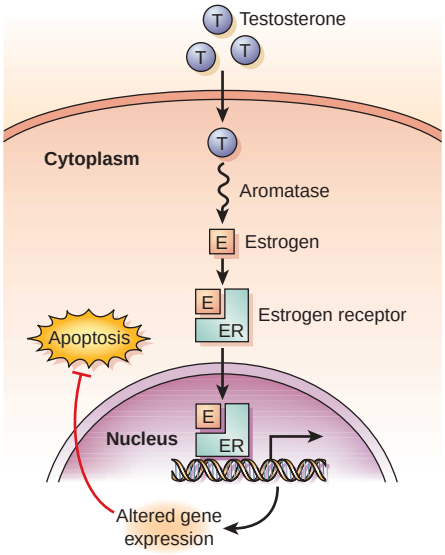

Figure 2 The conversion of testosterone into estrogen. In male rats, the testes secrete testosterone, which is converted into an estrogen in the brain by the enzyme aromatase. Estrogen then binds to estrogen receptors (ER) to modulate gene expression such that the SDN-POA differentiates in a masculine fashion. Steroid reduces naturally occurring cell death, resulting in more neurons surviving in males than in females.

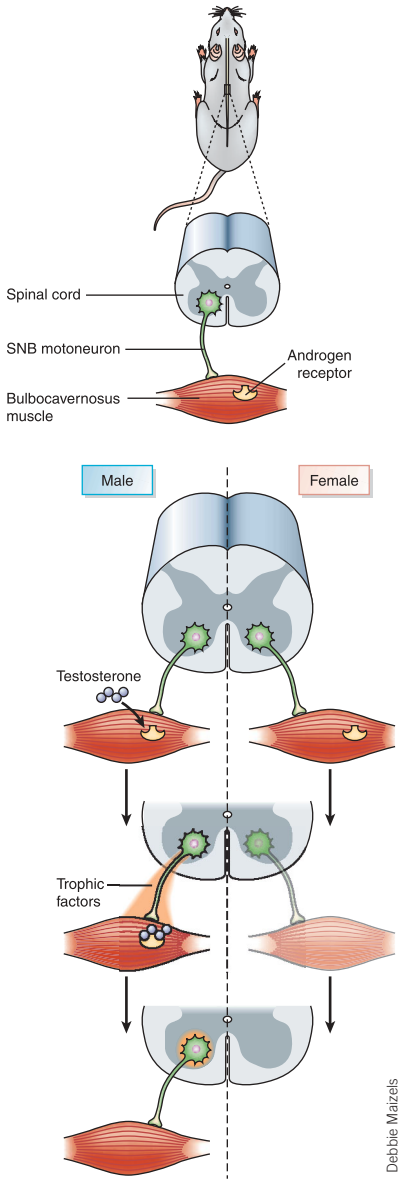

Figure 3 Before birth, the SNB system is present in both male and female rats, and motor neurons have established a functional neuromuscular junction. However, the muscles and their motor neurons die shortly after birth unless exposed to (or treated with) testosterone. In males, testosterone acts primarily to prevent the muscles from dying and this action secondarily prevents death of the motor neurons. One hypothesis is that testosterone induces the muscles to produce a trophic factor that preserves the muscles and either the same factor or an additional factor preserves the motor neurons.

this sex difference. Castration of adult male rats causes MePD volume and cell soma size to shrink to feminine proportions within 30 days after surgery and concurrently reduces male arousal to airborne cues from receptive females[22,24]. Conversely, treating females with testosterone for one month enlarges the MePD to masculine size[22].

Testosterone also affects the SNB system in adulthood: castration of adult males, though it does not affect the number of motor neurons or muscle fibers, nevertheless causes both to shrink[25], leading to a loss of spinal reflexes of the penis that are crucial for reproduction[3]. Testosterone treatment prevents this shrinkage by acting on androgen receptors[26,27], and this modulation of the SNB system probably reflects the seasonal breeding strategy of the ancestors of laboratory rodents, in which the reproductive system regressed each fall. For example, both the SNB system and the volume of MePD in male Siberian hamsters wane as reproductive systems are suppressed by short winter-like periods of light exposure in the laboratory, and will wax full again when the animals return to a reproductive condition[28,29]. Furthermore, MePD volume and cell soma size respond to exogenous testosterone in castrated hamsters only if they are kept in long summer-like periods of light exposure[30], so it appears that day length can regulate whether the brain will respond to testosterone.

Songbirds open up new possibilities

In several species of songbirds, males sing more than females, and the forebrain regions controlling song, including the higher vocal center (HVC) and the nucleus robustus archistriatum (RA), are larger in males than in females (**Fig. 1e,f**)[31]. In canaries, testosterone treatment of adult females, while insufficient to fully masculinize HVC and RA morphology, nevertheless enlarges these nuclei enough to induce the females to sing[32]. This remarkable level of plasticity in adult canaries includes the capacity to produce new, functional neurons[33], and it probably evolved in response to a reproductive strategy of seasonal breeding in which males compete, via the size of their song repertoire, for the most attractive females.

Zebra finches, in contrast, form longer-lasting pair bonds and retain reproductive capacity year-round. Accordingly, castration of adult male finches reduces singing only modestly, and testosterone treatment of adult females cannot induce them to sing nor their brain regions to grow[34]. The first studies of sexual differentiation of the zebra finch brain indicated a story very similar to that of mammalian dimorphisms. Treatment of newly hatched female zebra finches with estrogen, followed by testosterone treatment in adulthood, masculinized the females in terms of both singing and the volume of RA and HVC[35]. Furthermore, there are more dying cells in these regions in developing female zebra finches than in males, suggesting that estrogen prevents apoptosis in developing males, which results in a larger HVC and RA in adulthood[36].

But a perplexing problem emerged in zebra finches that does not fit the mammalian mold—neither castration early in life nor any pharmacological blockade of steroid receptors prevents these nuclei from developing a masculine phenotype in genetic males[37]. This difficulty in preventing masculinization of the brain of males gave rise to an alternative view: perhaps the genetic sex of the brain was directing masculinization independent of the gonads[38]. Experimental support for this idea came when researchers examined isolated slices of young zebra finch brain and found that differentiation proceeded according to the genetic sex: fibers from HVC entered and innervated RA more extensively in slices from males than from females. What's more, the brain slices produced detectable levels of estrogen in the medium, with slices from males producing more steroid than slices from females[39]. So it appears that a male genotype causes the zebra finch brain to locally produce steroid hormones, which then masculinize the birdsong system (**Fig. 4**). Consistent with this idea is a rare, spontaneously mosaic zebra finch, genetically male on one side and genetically female on the other, that had a larger song system on the male side than the female side[40]. There was even some indication that steroids derived from the male side of the brain might have diffused to masculinize the opposite side, as the brain regions of this animal's female side were slightly larger than that of control, wholly female brains.

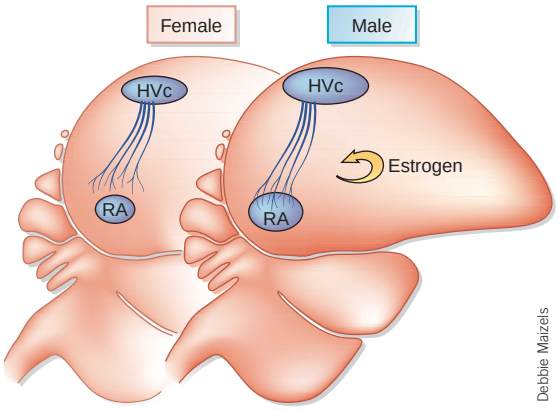

Figure 4 In newly hatched zebra finches, axons from HVC reach the vicinity of their target in RA, but do not actually enter RA in significant numbers unless estrogen receptors are stimulated. For example, treating young females with estrogen promotes axonal ingrowth into RA and a large, masculine RA in adulthood. But interfering with gonadally produced estrogen in males does not prevent masculine development, so it has been hypothesized that the genetically male zebra finch brain may produce its own estrogen to induce HVC ingrowth and masculine development. Explants of zebra finch brain confirm this idea, as slices from males release more estrogen into the medium than do slices from females[39].

The emerging recognition that genetic sex may directly masculinize the songbird brain has given rise to speculation that perhaps genetic sex also directly masculinizes the mammalian brain[41]. However, examination of mice in which genetic sex has been dissociated from gonadal sex indicates that the gonads direct sexual differentiation of mammalian neural structure and behavior. XY mice with a defective *Sry* gene develop ovaries, whereas XX mice carrying a transgene for *Sry* on an autosome develop testes. For the most part, these mutants confirm that it is the presence of testes, not the sex chromosome composition, that ensures a masculine central nervous system in terms of behavior, the SNB and other neural dimorphisms[42,43]. In a few instances, XX mice with testes, while more masculine than normal females, are not as masculine as XY male controls[43]. However, XX testes are demonstrably abnormal in some regards (*e.g.*, they do not produce sperm), so it is possible that the XX testes may not produce normal male levels of testosterone throughout all the sensitive periods for various brain regions, which would cause some regions to be under-masculinized. It is technically difficult to measure fetal testosterone levels in these animals, which would be required in order to rule out this possibility. To show that a gene affects neural sexual differentiation independently of the gonads, the ideal experiment would be to compare the brains of XX and XY mice that are genetically prevented from developing any gonads at all. If genes on the sex chromosomes directly drive brain sexual differentiation independent of the gonads, the brains of XY mice should be more masculine than those of the XX mice. If testes fully control masculinization of the mammalian brain, then the central nervous systems of gonadless mice, whether they carry XX or XY chromosomes, should be equally and fully feminine.

Neural sexual dimorphisms are remarkably diverse. Some rely solely on perinatal actions of testosterone (SDN-POA, AVPV), and some require both perinatal and adult testosterone (septal vasopressin, SNB, RA). Yet others require testosterone only in adulthood (MePD). In some cases, testosterone acts on only estrogen receptors (SDN-POA, AVPV) or activates both androgen receptors and estrogen receptors (septal vasopressin, MePD, RA). In other cases, only androgen receptors act perinatally (SNB). The zebra finch song system offers a distinctive twist: the brain itself seems to produce the 'testicular' hormone. But the commonality in all of these systems is that a single hormonal signal, testosterone, induces the central nervous system to take on a male phenotype—in the absence of testosterone, the nervous system differentiates as a female.

Where does testosterone first act?

In none of the cases described so far do we know the primary cellular targets on which testosterone acts to masculinize the nervous system during development. Although we know that exposing newborn female rats to estrogen prevents apoptosis of neurons in the SDN-POA, we do not know where estrogen acts to accomplish this. Is it directly on SDN-POA neurons themselves, or on other cells that then spare the SDN-POA neurons? That testosterone could act on distant target cells to spare SDN-POA neurons is entirely plausible because we know that testosterone acts on target muscles to spare SNB motor neurons. Likewise, no studies narrow down the list of possible targets for testosterone to masculinize the AVPV, MePD, RA or vasopressinergic innervation of the septum. Even for the SNB system, where we know that the developing motor neurons are not the site of action of testosterone, we do not know which cell types within the target muscles (muscle fibers, fibroblasts, Schwann cells) are directly modulated by the hormone.

However, we do know where testosterone acts to masculinize some systems in adulthood. Local implants of testosterone in the brain masculinize the morphology of ipsilateral birdsong regions in white-crowned sparrows[44] and in prepubertal zebra finches[45], so apparently testosterone acts somewhere within the forebrain in these cases. In genetically mosaic rats, only SNB motor neurons possessing a functional androgen receptor gene expand their somata or suppress peptide expression in response to adult testosterone treatment[26,46]. These cell-autonomous responses of SNB motor neurons are the only demonstrations to date of steroids directly altering the morphology or gene expression of neurons *in vivo*.

There are several genetic strategies that could provide information about the sites where steroids act to masculinize the developing nervous system. Once we understand whether testosterone masculinizes a system by activation of androgen receptor, or via aromatization and activation of either of two estrogen receptors, then it should be possible to conditionally knock out the gene for the relevant steroid receptor in particular classes of cells. For example, if the SDN-POA develops in a masculine fashion in males in which the estrogen receptor genes have been knocked out in astrocytes, but fails to differentiate properly in males missing estrogen receptor genes in neurons, then we will know that estrogen is acting specifically on neurons, perhaps even in the SDN-POA itself, to masculinize the nucleus. Similarly, if the SNB system is spared in genetic males that possess a functional androgen receptor only in muscle fibers, then the steroid must act on muscle fibers themselves to spare the muscles and their motor neurons.

Which downstream genes does testosterone modulate?

Because we do not know the target cells that first respond to testosterone or its metabolites during development, we know very little about the changes in gene expression that begin the process. In the SNB system, testosterone may alter the expression of trophic factor genes to spare the muscles and their innervating motor neurons. For example, injecting ciliary neurotrophic factor (CNTF) into the perineum of newborn rats spares the SNB system in normal females[47] and in males with a dysfunctional androgen receptor gene[48], indicat-

ing that CNTF is the molecular messenger dispatched by testosterone to maintain the system. However, male mice lacking the CNTF gene nevertheless develop a masculine SNB[49]. On the other hand, male mice lacking the gene for the CNTF receptor alpha subunit fail to maintain the SNB system[50], suggesting that some ligand(s) other than CNTF itself normally stimulates CNTF receptor alpha to maintain SNB muscles and motor neurons[51].

Although no genes have been implicated in the masculinization of the SDN portion of the POA, testosterone does induce the formation of prostaglandin E_2 (PGE_2), which then masculinizes another aspect of the POA, the formation of dendritic spines. These responses are also important for the development of male copulatory behaviors[52]. It is interesting that pharmacological manipulations of PGE_2 that affect spine formation in newborns (blocking PGE_2 in males stunts spine formation, whereas providing exogenous PGE_2 in females promotes spines) has no effect on the volume of the SDN-POA. This suggests that testosterone simultaneously starts several balls rolling: one that regulates expression of genes to produce PGE_2 (ref. 53), which then promotes spine formation, and another that regulates some other gene(s), which then acts to enlarge SDN-POA volume.

Sexual differentiation of the human brain

If steroids have such a pivotal role in masculinizing the brains of nonhumans, do they also have the same function in our own species? Do men and women behave differently because males are exposed to more testosterone prenatally? Do they behave differently because both sexes are indoctrinated into gender roles by family and society at large? The complexity of human behavior, which is powerfully shaped by social influences, makes it difficult to answer this question. On the surface, testosterone is responsible for sex differences in human behavior, if only indirectly—testosterone provides male fetuses with genitalia that provoke other people to raise them like boys. The deeper question is whether prenatal testosterone also masculinizes the fetal brain directly, without relying on other humans to shape the brain through experience.

Roughly 1 in 2,000 girls is exposed to slightly elevated levels of testosterone prenatally. Congenital adrenal hyperplasia (CAH) causes the fetal adrenal glands to produce androgens such as testosterone that slightly masculinize the genitalia and thus could potentially masculinize the fetal brain. Although these girls are more likely than other girls to engage in male-typical play[54], as adults most women with CAH are heterosexual. On the other hand, women with CAH are more likely to report a homosexual orientation than are other women[55]. However, because it is possible that the parents or the girls themselves may have ambivalent feelings about their 'true' gender, it is difficult to know whether these slightly masculinized behaviors are the result of prenatal testosterone inducing the brain to rebel against socially prescribed gender roles.

Another human syndrome suggests that if testosterone masculinizes the human fetal brain, it does so by acting on androgen receptors, not through aromatization and action on estrogen receptors. Genetically male (XY) individuals with a dysfunctional androgen receptor gene develop testes that secrete testosterone, but the body exterior, without a functional androgen receptor, develops a feminine phenotype. People with complete androgen insensitivity are usually undetected at birth because they appear to be girls, are raised as girls and as adults self-identify as women[56,57]. The feminine behavior of these women could be due to either the brains' inability to respond to fetal testosterone, or their rearing as females. Thus, this syndrome does not tell us whether social influences or prenatal steroids inculcate sex differences in human behavior. But if, as in rats, testosterone masculinized the brain of fetal humans by being converted to estrogens and activating estrogen receptors, then we would expect these people with women's bodies to display male behaviors, because their estrogen receptor genes are intact.

An important but elusive question is whether prenatal steroids masculinize the human brain with respect to sexual orientation. Does the testosterone that masculinizes the human body also masculinize the fetal brain so that, in adulthood, the person will be attracted to women? One strategy has been to find somatic markers that correlate with prenatal androgen and ask whether they also co-vary with sexual orientation. Eyeblink patterns[58], otoacoustic emissions (clicks emanating from the ears)[59] and measures of fingers[60] and limbs[61] all indicate that lesbians, on average, are exposed to more prenatal androgen than heterosexual women. However, none of these studies indicate a simple relationship between prenatal testosterone and sexual orientation—there is considerable overlap between lesbians and heterosexual women for each marker, which makes it clear that testosterone is not the sole factor at work. Nevertheless, crude as these measures are, it is impressive that such a wide range of characteristics, reported by a wide number of laboratories, all indicate that the more prenatal testosterone a girl is exposed to, the more likely she is to self-identify as lesbian in adulthood. Examining these same markers in men provides conflicting results, sometimes yielding evidence that gay men are exposed to less prenatal testosterone than straight men, sometimes finding the opposite and sometimes finding no differences between the two groups. While still controversial, it is possible that both lower-than-average and higher-than-average testosterone exposure before birth can increase the likelihood that a boy will develop a homosexual orientation.

Of course, animal models have helped by framing the questions that scientists have asked about the origins of human sexual orientation. For example, the models tell us that if we want to seek a prenatal influence on human sexual orientation, we should pay attention to testosterone and its target tissues in the brain. Studies of the rat SDN-POA inspired examination of the human POA, where a nucleus was found that was smaller in women than in men, and smaller in gay men than in straight men[62]. A similar difference is seen in sheep: a nucleus in the POA is smaller in male sheep that prefer to mount other rams than in males that prefer to mount ewes[63]. For neither humans nor sheep do we know whether the differences in the POA preceded the development of sexual orientation or arose after the establishment of orientation, but taken together, these studies make it seem likely that this brain region is important for determining sexual orientation. A better understanding of how these and other sexual dimorphisms in the nervous system arise will help us appreciate why men and women behave differently, and why most of us find one of the sexes, and only one, so very enchanting.

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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