
Sexual Differentiation of the Brain and Behavior: A Primer

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Abstract

A general theory of sexual differentiation of the brain derives from classic experiments performed in the twentieth century, which showed that androgens from the testes act early in the development to cause some regions of the male's

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brain to develop differently from those in the female. Further sex differences in brain structure and function are induced also by the effects of sex hormones from both the testes and ovaries later in life. In addition, sex chromosome genes on the X and Y chromosome are expressed inherently differently in female and male brains and cause them to be different. The sex hormones create specific sex differences in brain function by regulating cell death and birth leading to sex differences in the number of neurons. Sex hormones also regulate the length of dendrites and number of synapses devoted to specific tasks. Sex hormones cause epigenetic changes with broad impact on a large number of molecular pathways. A general principle is that cellular and molecular mechanisms leading to sex differences are highly diverse, differing in each specific brain region. Moreover, in humans the effects of gendered environments interact with multiple biological factors to produce brains that are not necessarily uniformly masculine or feminine, but a unique mixture in each individual.

Keywords

Sex hormones • Sex chromosomes • Cell death • Synaptogenesis • Epigenetic

Introduction

In sexually reproducing species, there are usually two sexes, female and male. Males are defined as the sex that makes small gametes called sperm, and females make larger gametes called eggs. These sex differences have led to the evolution of two different bodies, each adapted to producing one type of gamete and coordinating reproductive events (e.g., production and exchange of gametes, courtship, copulation, and rearing of young) so that the DNA of each sex is passed to the next generation. Sex differences in structure and function occur dramatically in the tissues involved with reproduction, such as the gonads, genitals (internal and external), and some parts of the brain. They also occur less dramatically in nonreproductive tissues, including areas of the brain that are not obviously involved in reproduction. The primary focus of this chapter is on the events throughout life that cause the two sexes to differ, physiologically and behaviorally. **Sexual differentiation** is defined as the process by which the two sexes develop differences in any trait. The word **differentiation** has two connotations that have caused some ambiguity of interpretation. From the field of developmental biology, “differentiation” refers to change during the lifetime of the individual. An undifferentiated cell or tissue loses pluripotency, commits to a specific fate, and changes into a different form. In that case, the differentiation is a change in the same individual over time. However, in the field of sexual differentiation, the term “differentiation” also means a difference in development between males and females. The second comparison therefore is between two different individuals,

rather than in the same individual at different times. According to the second connotation, any sex difference is the result of sexual differentiation, whether it involves irreversible commitment to a specific fate or not. The study of sexual differentiation includes finding the molecules that make one sex develop differently from the other sex, explaining how those molecules work and determining their significance in the lifetime of the individual.

Are molecules the only causes of sexual differentiation? In vertebrates such as mammals, and especially in humans, males and females are reared in different social or even physical environments. These different environments have important effects on biology, especially of the brain. Is it possible that the different environments of the two sexes also cause sex differences in biological development? Do gendered social attitudes affect our bodies? The answer is most certainly yes. However, in this chapter, the emphasis is on the biological (molecular) factors that make females and males different.

Why study sexual differentiation? Classically sexual differentiation was a sub-field of reproductive biology. Understanding the different development of males and females was important for appreciating the function of reproductive tissues. It also helped explain why these tissues sometimes develop abnormally. In recent years, however, scientists and physicians have increasingly realized that other parts of the body (e.g., liver, bone, fat cells, and nonreproductive areas of the brain) also show sex differences, both under normal conditions and in the face of disease. Consider the example of multiple sclerosis, a debilitating brain disease in which the immune system abnormally attacks the myelin sheath surrounding neuronal axons. Women suffer from multiple sclerosis 3–4 times more often than men, because of sex differences in their immune system and the brain's reaction to an immune attack. This means that men have some molecules that protect them from this disease. In contrast, boys are affected by autism spectrum disorders much more than girls, indicating that females are somehow protected. Our understanding of sexual differentiation helps to identify such sex-biased protective factors as part of a strategy for developing new therapies, for example new drugs that would enhance the protective factor and alleviate disease.

Sex differences come in all sizes. In some cases, male and female tissues have two distinct forms that do not overlap in size or function. The most obvious examples are the external genitals. Males have a penis and scrotum, and females have a clitoris and vaginal labia. These are so completely dimorphic (“di-” means two and “morph” means structure) and obvious that we use them as a primary indication of the sex of the individual. In the brains of animals, sex differences are sometimes as dimorphic as the genitals, but usually not. When the brain is found to be different in females and males, often the two sexes differ *on average*, with a lot of overlap. In such cases, many individuals of each sex have the same trait as individuals of the other sex. Because large and small sex differences are caused by the same factors, for simplicity in this chapter we will use “sexual dimorphism” as shorthand to refer to *any* average sex difference.

A Conceptual Framework for Understanding Sexual Differentiation

Early Experiments to Find the Causes of Sexual Differentiation

The first studies of sexual differentiation were aimed at explaining the largest sexual dimorphisms in the external genitals as well as the internal genitals (the uterus and oviducts and sperm ducts). In 1916, the embryologist Frank Lillie published a study of the intersex freemartin calf. When cows have twins, sometimes one twin is a normal, reproductive male, but the other is an intersex freemartin with a mixture of male and female structures such as female external genitals but some components of the internal male reproductive tracts. Lillie inferred that the sterile intersex twin was a genetic female and that the intersexuality occurred only when the blood of the male twin became mixed with that of the female twin before birth. The conclusion was that blood-borne substances from the male, now called hormones, were causing partial male-like development of the female (masculinization). Lillie thought that the hormones were coming from the testes. In the 1940s, the French endocrinologist Alfred Jost put Lillie's theories to a successful experimental test. He removed the gonads of male and female rabbit embryos and assessed how the external and internal genitals developed. If the testes were removed early enough from a genetic male rabbit, it developed female external and internal structures, such as vaginal labia, uterus, and oviducts. Those experiments confirmed that the testes are the origin of hormones that normally make these structures male or block the female form. However, Jost found that removing the ovaries from a genetic female had no effect and that she developed normal female external and internal genitalia. These experiments established an asymmetry in sexual differentiation, in which the male's testes secrete hormones that make his body different from that of females, but the female's ovarian hormones are not required to make her different from the male. Because the female form developed even when ovarian hormones were absent, Jost called the female pattern of development "default," the pattern that developed in the absence of hormonal effects. Jost established that a critical testicular hormone was testosterone, because treating female embryos with testosterone caused male development of the external genitals, the penis and scrotum. He also discovered that the testes secrete a second hormone, called Müllerian-inhibiting Hormone (or anti-Müllerian hormone), which blocks the development of female internal genitalia such as the uterus and oviducts.

At the same time (mid-twentieth century), behavioral biologists were trying to explain sexual differentiation of behavior. For example, when rodents are in reproductive condition, males will mount a female during copulation. Rather than mounting, females show lordosis behavior in which they arch their back, elevate their rump, and provide the male access to their vaginal area, thus allowing copulation. Experiments of pioneers such as Frank Beach demonstrated that the male's behavior was stimulated by testosterone from the testes, and the female's behavior was stimulated by estradiol and progesterone from the ovaries. However, even if a male is injected with estradiol and progesterone as an adult, he will show relatively

little lordosis behavior. Similarly, giving testosterone to adult females does not induce them to mount other females as much as males. Thus, the male appears to lack the behavioral capacity of the female, and the female lacks the capacity of the male. Similarly, in tests of reproductive aggression, males will attack a male cage mate, especially if the attacker has elevated levels of testosterone. Females show much less aggression in similar testing, even if they are treated with testosterone as adults. Thus, the female lacks the behavioral capacity of males. Although females can be aggressive, they show that aggression in other situations such as when protecting their pups.

These studies indicated that some factor other than the levels of hormones circulating in adulthood was needed to explain sex differences in reproductive and aggressive behavior of adult animals. The ideas of Lillie and Jost provided a possible answer: perhaps brain sexual differentiation was analogous to genital sexual differentiation. The hypothesis was that sex differences in behavioral capacity were sexually differentiated prenatally by testicular secretions, just as testosterone acts to cause embryonic male external genitals to develop differently than those of the female. To test this idea, an experiment was performed in 1959 by Charles Phoenix, Robert Goy, Arnold Gerall, and William Young. They injected pregnant guinea pigs with testosterone, which reached the female fetuses. When these androgenized females reached adulthood, they showed less lordosis (less female behavioral capacity) when placed with a male partner, compared to females that were not androgenized before birth. The androgenized females also showed more male mounting behavior, when placed with a female partner, relative to females not exposed to androgen prenatally. Other experiments, which removed testes from males or blocked testosterone action, showed that the embryonic and newborn male's testes produce testosterone, and it is this source of hormone that acts to make behavioral capacity more like that of a male (masculinization) and less like that of a female (defeminization).

A Hormonal Theory of Brain Sexual Differentiation

In their classic 1959 paper, Phoenix et al. introduced two terms that have had lasting value in conceptualizing sexual differentiation. They believed that before birth, the male's testes secrete testosterone, which acts somewhere, probably on the brain itself, to cause permanent changes in behavioral capacity. They considered this process to be conceptually similar to the morphological differentiation of the penis, in that both are masculinization caused by testosterone. However, Phoenix et al. were not actually measuring the structure of the brain, but rather just imagined that something was changed there (an alteration of the *substrate*) which was permanent (as evidenced by the permanent masculinization of behavioral capacity throughout adulthood). However, the same hormone, testosterone, also acted later in life on the changed substrate to activate the reproductive behaviors they were measuring. The two phases of testosterone action were given distinct names. The actions of testosterone prenatally were called *organizational effects*, which were

differentiating and permanent. The later actions of testosterone were called **activational effects**, because they activated the presumptive differentiated substrate. Activational effects were reversible, because the effect of testosterone to activate mounting behavior of adult males only lasted as long as the testosterone was present, in contrast to the organizational effects that were considered to last forever. Classically, the organizational effects are differentiating, especially in the first sense of the word “differentiation” (permanent change from an earlier developmental state). Considered from the viewpoint of the second meaning of “differentiation” discussed above, both types of effects of testosterone cause the male to be different from the female, so are both sexually differentiating.

Since 1960, the organizational-activational dichotomy, and theory of sexual differentiation, has been applied extensively to understand sex differences in many different animals, in a wide variety of tissues and behaviors. In general, the theory has been very useful and has passed many experimental tests. If one discovers a trait that differs in females and males, the organizational theory is tested in two ways. Females are administered testosterone early in life (prenatally or just after birth) and compared to control females who are treated with a placebo. The androgenized females are found to be more masculine or less feminine as adults compared to control females not exposed to androgens (here, “masculine” refers to whatever traits are typical of males, and “feminine” refers to whatever traits are typical of females). Alternatively, males are deprived of testosterone early in life and found to be less masculine or more feminine in that trait later in life. In contrast, the idea of hormonal activation is tested in adult animals, by reducing or increasing the levels of testicular or ovarian hormones to determine if they cause one sex to be different from the other.

One puzzling finding emerged in the 1960s, which was that treating female rodents with estradiol, just after birth, mimicked the permanent organizational effects of testosterone. As adults, such females showed more male behavior, not less, compared to control females. They also showed less female behavior rather than more. How could treatment with a female hormone make females more like males? The resolution of this paradox came with the understanding that when testosterone is secreted by males pre- and postnatally, it enters the brain where it is converted to estradiol by the enzyme aromatase. Estradiol is actually the active hormone in males, which acts within the brain to cause masculinization and to block development of female functions and behaviors. Therefore, it is incorrect to think of estrogens only as hormones of females. In reality, both males and females produce androgens and estrogens, which naturally play specific functions in both sexes. However, adult males generally have higher levels of androgens than adult females, and adult females have higher levels of estrogens than adult males.

Another paradox was that if estradiol has a masculinizing effect, then why are females not masculinized by estradiol from their own ovaries or from the ovaries of their mother during gestation? This paradox was resolved by the discovery that a liver protein, alpha fetoprotein, is secreted into the blood of rodents before and after birth. There it binds estrogens and prevents them from entering the brain to cause masculinization. In other words, the alpha fetoprotein acts like a sponge that sequesters the hormones in the blood so that it cannot gain access to the brain and

induce masculinization. Experiments show that genetic mutation (knock out) of the alpha-feto-protein gene results in significant masculinization of the brain of female mice, confirming that sufficient estrogen is present in the womb to cause masculinization of the brain and behavior, and that this is prevented by alpha-feto-protein.

Thus, sexual differentiation of the brain has long been attributed primarily to the developmental masculinizing effects of estradiol in rodents. Testosterone, secreted by the fetal or neonatal testes, acts as an androgen on the genitals to cause masculinization but is converted to estrogen in the brain to cause masculinization. In humans, it is thought that the conversion to estrogen is much less important and that the permanent masculinizing effects of testosterone, both on the genitals and the brain, do not require conversion to estrogens. However, there remains the possibility of an as yet undiscovered role for estrogen in human brain development.

The permanent masculinizing effects of testosterone and estradiol are limited to specific critical periods of development. For example, for the external genitals to form into a penis and scrotum, testosterone must be present during the middle and/or late periods of gestation of the embryo and fetus, when genital differentiation occurs. Treating females with testosterone before birth masculinizes their genitals, but not if they are treated after birth. Similarly, specific brain regions or behavioral circuits pass through critical periods during which testosterone or estradiol acts to masculinize them. If testosterone or estradiol is elevated outside of the critical period, it has no permanent organizational masculinizing effect. Because numerous brain regions and behavioral-physiological functions are mediated by different parts of the brain or other tissues, there are multiple critical periods attributed to each brain region or function. Thus, injecting a female rat with testosterone or estradiol after birth masculinizes her copulatory and other behaviors, but not her external genitals because the genitals have passed through their critical period of masculinization by testosterone.

Towards a Cellular-Molecular Appreciation of Brain Sexual Differentiation

Several events in the period 1970–1990 moved the field of brain sexual differentiation beyond behavioral measurements, to measurement of cells, tissues, and molecules. Large structural sex differences in specific brain regions were discovered (Fig. 1), in brain regions controlling reproduction. In songbirds, for example, males sing a courtship song to attract females and repel males. In many species, females generally cannot produce this male-typical song, or they produce a song of lesser complexity. Song is controlled by an interconnected set of discrete brain regions, which can be seen clearly under the microscope. Fernando Nottebohm and Arthur Arnold in 1976 reported that several of the brain regions were as much as five or six times larger in males than in females and that there was little or no overlap in the size of brain regions in the two sexes. Shortly thereafter, Roger Gorski and colleagues reported that the preoptic area of the rat hypothalamus contained a cluster of cells that was also much larger in males than in females. The preoptic area is generally

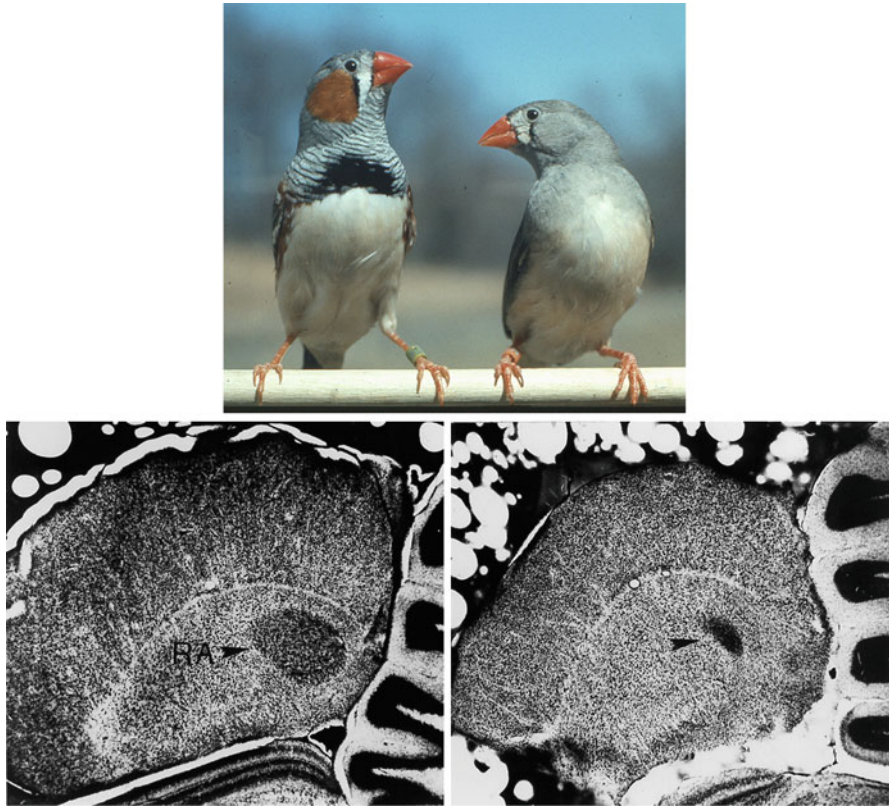


Fig. 1 Large sex differences in the brain of songbirds. In the Australian zebra finch (*above*), a desert-dwelling species that is a popular cage bird around the world, the male (*left*) sings a courtship song to the female, which the female cannot sing. In the brain is an interconnected series of regions that control song and song learning, which are much more developed in males than in females. The robust nucleus of the arcopallium, below, is 5–6 times larger in males than in females

implicated in the control of male copulatory behavior. Other morphological sex differences were reported in the hypothalamus, lateral septum, and spinal cord of the rat. These discoveries meant that investigators could begin to focus on specific brain regions that were known targets of the sexual differentiation process to determine mechanisms by which sex-biasing forces induce sexual differentiation.

Another critical development was the identification of specific receptors that mediate the effects of sex steroid hormones such as androgens and estrogens. These hormone receptors must be expressed in specific cells if the cells are to respond to testosterone or estradiol, respectively. Although sex steroid hormone receptors in the cell's nucleus were the first to be identified, it is now known that each sex steroid hormone acts on two or more different receptors, which are in the nucleus or inserted into the cell membrane. Once the hormone binds to the receptors, it triggers a chain of molecular reactions that cause changes in cell function, discussed

further below. The identification of molecules required for sex steroid hormone action helped to revolutionize the study of sexual differentiation. The sites of sex hormone action were uncovered partly by discovering cells that express the required receptors. Moreover, inactivation of the receptors, using drugs that antagonize hormone action, or mutation of specific receptor genes, became important tools for discovering the cellular sites and mechanisms of sex hormone action in the brain and other tissues. In all cases, the brain regions that respond to sex steroids during sexual differentiation must express the appropriate receptor (e.g., either androgen receptors or estrogen receptors, or both) or to be influenced by other cells that do.

The discovery of cells that are direct targets of estrogens or androgens, and which were found to be sexually differentiated because of the permanent action of testosterone or estradiol, allowed further tests of the organizational-activational theory at the level of cells rather than at the level of behavior. Consider three sexually dimorphic regions of the central nervous system: SDN-POA, the sexually dimorphic nucleus of the preoptic area of rats, which is larger in males than in females and regulates male sexual behavior; AVPV, the anteroventral periventricular nucleus of the hypothalamus, larger in females than in males, which controls ovulation; and SBN, the spinal nucleus of the bulbocavernosus in rat spinal cord, which is larger in males than in females and controls muscles of the penis. In these neural regions, treatment of neonatal females with testosterone or estradiol caused permanent masculinization or defeminization (larger SDN-POA, smaller AVPV, larger SNB). Those findings confirmed the permanent sexual differentiation of the neural substrate imagined in 1959 by Phoenix et al. Moreover, blocking estrogen or androgen action in neonatal males prevented masculinization and promoted feminization (smaller SDN-POA, larger AVPV, smaller SNB). The potent effects of sex steroid hormones fully accounted for major brain sex differences and led to the belief that these were the only molecules that initiate sexual differentiation of brain structure and function. The organizational-activational theory was therefore found to be exceptionally useful for explaining diverse sex differences in brain function, structure, and behavior.

Sex Chromosome Effects

From the earliest years of the twentieth century, even before the discovery of organizational hormone effects, a major question was whether sexual phenotypes (traits that differ in the two sexes) were caused by genetic or hormonal effects. In each species with heteromorphic sex chromosomes (sex chromosomes that differ in size), the two sexes have a different set of sex chromosomes and sex chromosome genes. In mammals and *Drosophila* fruit flies, males have XY sex chromosomes and females have XX. In birds, males have ZZ sex chromosomes and females ZW. Thus, male mammals have Y chromosome genes not found in females and females have two X chromosomes rather than the single X of males. These sex differences in the genome are present from the first day of life, when the fertilized egg cell (zygote) begins its differentiation into a whole individual. The ideas of Lillie that sexual

differentiation in mammals was caused by hormonal secretions contrasted with the results from genetic research in *Drosophila* that showed that the sex of the fly was determined by the complement (number and type) of sex chromosomes. Ultimately it was decided that sexual differentiation of invertebrates is largely genetic, but sexual differentiation of vertebrates is largely hormonal.

By the late 1930s, a dominant two-stage theory of sexual differentiation of mammals emerged. In mammals, the differentiation of the gonads was thought to be the first stage of sexual differentiation, called “sex determination.” Sex determination was considered to be genetic. This was confirmed conclusively by 1959, when the presence of the Y chromosome in mammals was shown to be required for the development of testes, and ovaries developed under the control of factors that are expressed when a Y chromosome is absent. The most important male-determining gene on the Y chromosome is *Sry*, which causes testes to develop. The number of X chromosomes was found not to affect the sex of the gonad. Once the gonads formed, however, they secreted different hormones in the two sexes, causing stage two or “sexual differentiation” of the rest of the body. The two-stage idea, genetic sex determination, followed by hormonal sexual differentiation, accounts for many experimental findings. However, it is incomplete because sex differences in nongonadal tissues emerge well before sexual differentiation of the gonads. In addition, the inherent imbalance of X and Y gene action throughout the body has been found to cause sex differences in diverse adult tissues including the brain.

The two-stage hormones-only theory was criticized for several reasons. Some sex differences in the brain were difficult to explain by the hormonal theory. Consider the zebra finch, in which brain regions controlling courtship song are much larger in males than in females (Fig. 1). Experiments were performed to test the organizational-activational hormonal theory in this system. Various procedures, which blocked the action of androgens and estrogens in males early in development, failed to prevent masculine development of males. Two experiments, conducted in the lab of one of the authors (Arnold), were particularly difficult to reconcile with the idea that gonadal secretions account for all sex differences in the song system. Drug treatments, which caused testes to develop in genetic females, failed to cause masculine development of the song system. And investigation of an intersex gynandromorphic zebra finch suggested that sex chromosome genes might contribute to sexual differentiation. The gynandromorph (“gyn” for female, “andro” for male, and “morph” for form) had male plumage on the right side of its body, and female plumage on the left, divided down the middle of its body (Fig. 2). On the right was a testis and on the left was an ovary. Genetic tests of this bird and other gynandromorphs indicated that it was genetically male on the right side and genetically female on the left. Therefore, this bird provided the perfect chance to test the idea that sexual differentiation of the brain song network was caused by hormones coming from the gonads or alternately by sex chromosome genes residing within brain cells themselves. If hormones were causing sexual differentiation, then the brain regions would be expected to have the same sexual phenotype on both sides of the body, because

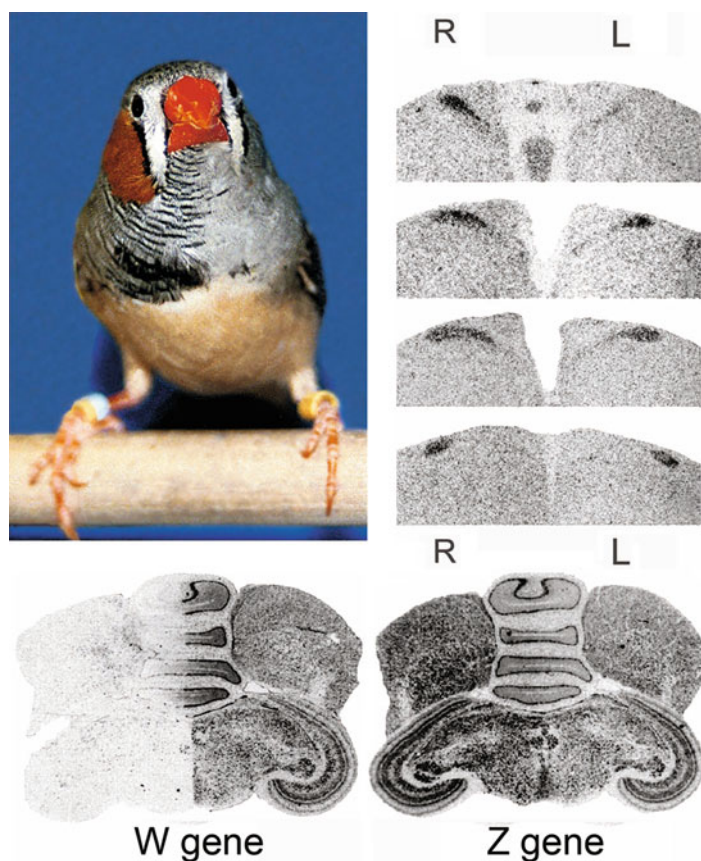


Fig. 2 A half-male, half-female zebra finch. Rarely are lateral gynandromorphs discovered in birds. This bird (*upper left*) had male plumage on the right side of the body, with a sharp dividing line down the middle, with female plumage on the left side. The right gonad was a testis, and the left an ovary. In the lower left panel, in situ hybridization shows dark areas where a W gene is expressed on the left side. Because W genes are normally found only in females, the expression suggested that the left side was genetically female. Z genes, expressed from two Z chromosomes in ZZ males, rather than one Z chromosome in ZW females, were expressed in the gynandromorphic finch at a higher level on the right, genetically male, side of the brain (*lower right*). The upper right panel shows brain sections of song control nucleus HVC, stained dark by the heavier expression of the androgen receptor. HVC is much larger in normal males than in normal females and was larger on the genetically male (*right*) side of the brain of the gynandromorph, compared to the genetically female (*left*) side of the brain. Because the size of the HVC correlated with genetic sex of the brain cells, instead of the hormonal levels inferred to be equivalent on the two sides of the brain, the study of the gynandromorph suggests that the genetic sex of cells contributes to sex differences in brain traits (From Agate et al., *Proceedings of the National Academy of Sciences USA*, 2003 v100, page 4873)

gonadal hormones would affect both sides. On the other hand, if sex chromosome genes act to cause brain regions to be masculine or feminine, then the song system on the right side should be masculine, but on the left should be feminine. Histological analysis of this bird showed that the right side was more masculine than the left, providing evidence that the genetic sex of cells contributes to brain sexual differentiation (Fig. 2). Further analysis of gynandromorphic chickens showed similar right-left differences in sexual phenotype throughout the body, explained by the lateral difference in genetic sex of cells.

In mammals, too, XX cells might be different from XY cells not only because of the hormones that act on them from the outside, but also because of endogenous actions of X and Y genes within the cells themselves. These genes are the only ones that are inherently different in XX and XY cells, even before the gonads develop. To test this idea, it is necessary to change the sex chromosomes and measure the effects on the phenotype of cells and tissues. For example, one might try to give an XX mouse a Y chromosome to determine if the Y chromosome changes the brain. That creates a problem, however. Comparing mice with and without a Y chromosome involves not only differences caused by Y genes in the brain, but also differences in the effects of sex hormones because mice with a Y have testes, but mice without a Y have ovaries. How does one change the sex chromosomes, to measure their effects, without also changing the hormones? One solution is to remove *Sry*, the gene that makes testes develop, from the Y chromosome, to produce a Y^- chromosome. XY^- mice have ovaries rather than testes. That allows one to compare XX and XY^- females (called XXF and XYF) to see if mice differ when they have different sex chromosomes but the same type of gonad.

The Y^- chromosome is one of the features of a mouse model called the "Four Core Genotypes" (FCG). The father of FCG mice has XY^- sex chromosomes but also an *Sry* transgene inserted into chromosome 3, not a sex chromosome (Fig. 3). The *Sry* transgene compensates for the loss of *Sry* from the Y^- chromosome and makes the mouse a gonadal male. Mating such XY^- (*Sry*+) males (called XYM) with XXF gives four different types of babies, the four core genotypes: XXF, XYF, XXM, and XYM. To determine if the sex chromosome complement (number and type of sex chromosomes) contributes to a sex difference in a trait, one measures the trait in XXF compared to XYF, or in XXM compared XYM. One can also measure the effects of hormones by comparing mice with testes (with *Sry*) (XXM and XYM) to mice with ovaries (XXF and XYF).

Studies of FCG mice show that sex chromosomes can have large effects (Fig. 4). In C57BL/6 inbred mice, adult males weigh about 25 % more than females, no matter what their sex chromosome complement. When the gonads are removed from FCG mice, this sex difference goes away. A month after removal of the gonads, males and females weigh about the same. However, slowly thereafter a large effect of sex chromosome complement emerges. XX mice, both male and female, eventually weigh up to 25 % more than XY mice (either male or female). The removal of gonadal hormones therefore unmasked an effect of sex chromosomes, which can produce a sex difference as large as that caused by gonadal hormones. Of course, the sex chromosomes were present at all ages of the mice. Did they also have an effect when the

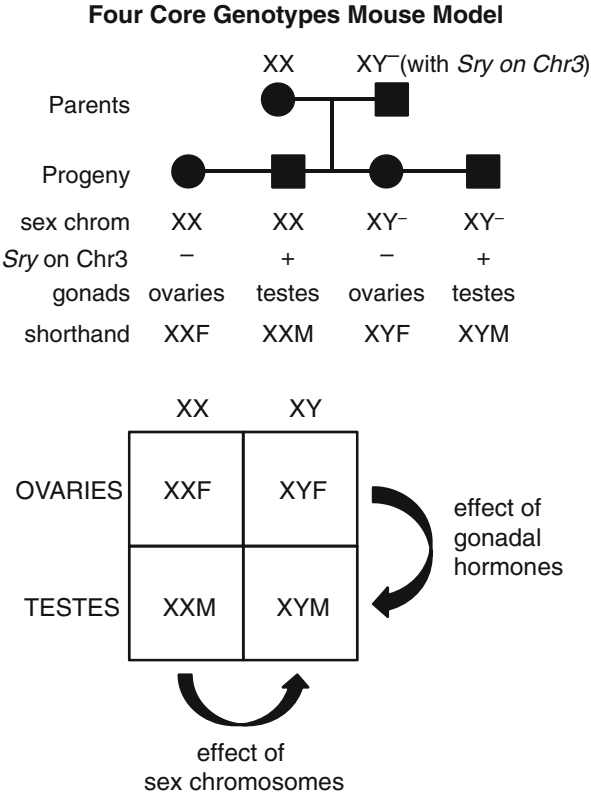


Fig. 3 The Four Core Genotypes mouse model. This model aims to separate the effects of sex chromosome complement (XX vs. XY) from the roles of gonadal hormones (testes vs. ovaries). The father has a variant Y⁻ chromosome deleted for the testis-determining gene *Sry*, plus an *Sry* transgene inserted onto chromosome 3. Because *Sry* and the Y⁻ segregate independently, the father makes four kinds of sperm (X or Y⁻, each with or without *Sry*). This results in offspring of four genotypes, XX with testes (XXM) or ovaries (XXF) and XY with testes (XYM) or ovaries (XYF). The model is a 2 × 2 comparison of the effects of sex chromosome complement (XX vs. XY) and of gonadal hormones (testicular vs. ovarian). The effect of gonadal hormones is seen when comparing traits of gonadal males and females (XXF vs. XXM, or XYF vs. XYM). The effect of sex chromosome complement (XX vs. XY) is seen when comparing XX and XY mice with the same type of gonad (XXF vs. XYF, or XXM vs. XYM)

gonads were present? Figure 4 shows that XX mice were heavier than XY mice even with the gonadal hormones present, but the effect was smaller than in the absence of gonads, about 8 %. The conclusion is that the gonadal hormones acted to reduce the effects of sex chromosomes, and vice versa. Because male hormones (from the testes) make the mouse weigh more, but male sex chromosomes make the mouse weigh less, it is clear that hormones and sex chromosomes counteract the effects of the other. Thus, independent sex-biasing factors sometimes *compensate* for the effects of each other and can reduce sex differences in a trait rather than enhancing them.

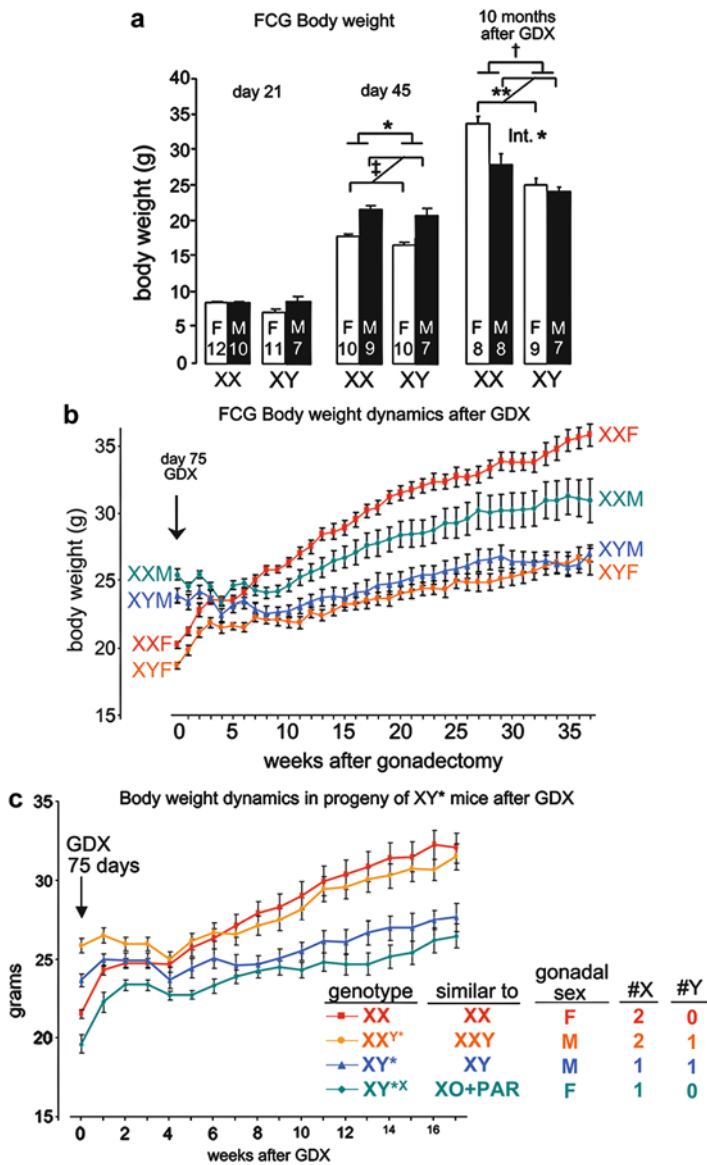


Fig. 4 Sex chromosome effects on body weight. (a) The body weight of FCG mice was studied when the mice were weaned at 21 days of age. At that point, the FCG groups have similar body weight. After the mice have experienced higher levels of gonadal hormones at the time of puberty (day 45), mice with testes had greater body weight than mice with ovaries ($\dagger p < 0.000001$), and mice with XX sex chromosomes weighed slightly more than XY ($*p < 0.05$). This result suggests that the main factors causing sex differences in body weight are gonadal hormones, but that sex chromosome complement plays a smaller role. To test the role of gonadal hormones, the mice were gonadectomized (GDX) at 75 days of age. Ten months later, XX mice weighed much more than XY mice, in groups with testes or with ovaries ($\dagger p < 0.0001$), and gonadal females were heavier than

The greater body weight of gonadectomized XX mice, compared to XY, is caused by a large increase in body fat. One of the main differences in these mice is that XX mice eat more during the light phase of the 24-h cycle, when they are normally inactive or sleeping. However, the greater amount of body fat is probably caused by a number of physiological differences in mice with XX or XY chromosomes.

A major question is whether the XX versus XY difference is caused by Y genes that reduce body fat or by a double dose of X genes found in XX mice that increase body fat. Comparisons of XO versus XX mice, and XY versus XXY mice, show that the number of X chromosomes makes the difference. Thus, one or more X genes are a factor that makes XX and XY metabolism different. In studies of other mouse models of disease, the Y chromosome is also found to cause differences between XY and XX mice. Thus, the number of X chromosomes and Y genes are established as primary factors causing sexual differentiation. They are primary because each is caused by an inherent difference in sex chromosome complement.

The major conclusion from the body weight study and other experiments is that sex differences are controlled by a complex interaction of different sex-biasing factors, both hormones and sex chromosomes. During the lifetime of the individual, any one of the sex-biasing factors can increase or decrease because of natural aging, circadian or other rhythms, or disease. Thus, the balance in effects of hormonal and sex chromosome factors can shift. For example, disease may reduce sex hormone levels, leading to greater differences in the effects of XX and XY genes.

The sex chromosomes contribute to sex differences in behavior and brain morphology. In addition, they help explain sex differences in various mouse models of disease, including autoimmune diseases such as multiple sclerosis and lupus, obesity and metabolism, cardiovascular disease, Alzheimer's disease, aging, and neural tube closure defects.

A General Theory of Sexual Differentiation

The above narrative discusses experimental findings over the last century and implicates several major classes of molecules as causal agents differentiating the two sexes. To mold these ideas into a single theory, we begin with the fundamental



Fig. 4 (continued) gonadal males (** $p < 0.01$). The effects of sex chromosome complement interacted significantly with the effects of gonad type (Int, * $p < 0.05$), because the effect of sex chromosome complement was larger in mice with ovaries than in those with testes. **(b)** Growth curves for FCG mice that were weighed at day 75 (week 0) and then gonadectomized (GDX). By 4–5 weeks after removal of the gonads, the sex difference caused by gonadal secretions in adulthood disappeared. After that time, XX mice gained much more weight than XY mice. **(c)** The sex chromosome effect on body weight is confirmed in the XY* model and found to be caused by the number of X chromosomes. The same GDX design was used as in *panel B*. After GDX at day 75, mice with two X chromosomes gained weight more than mice with one X chromosome ($p < 0.000001$) (From Chen et al., *PLoS Genet.* 2012, 8:e1002709)

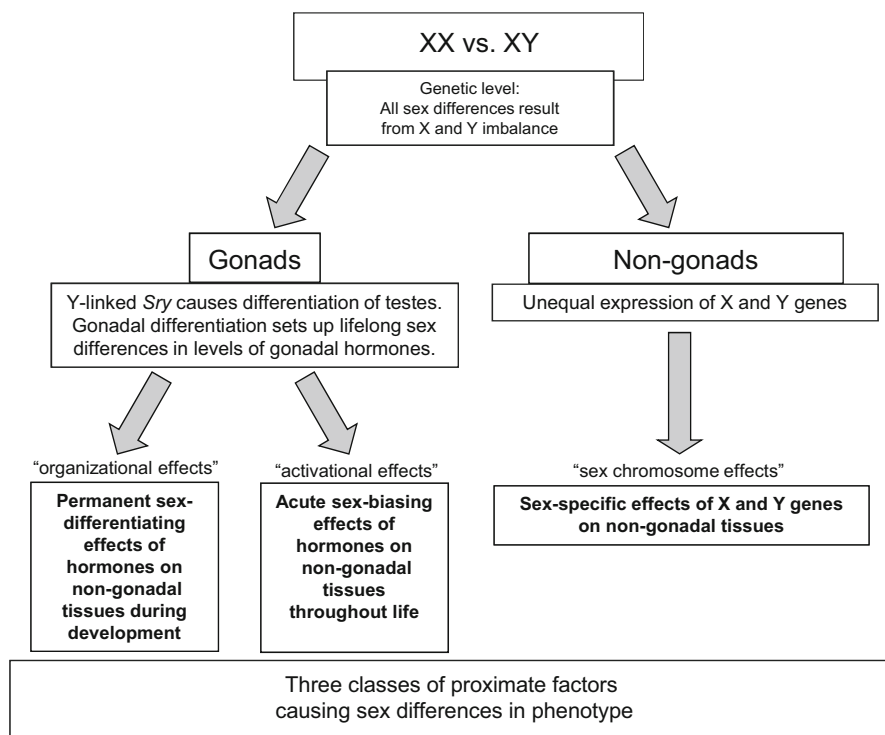


Fig. 5 A general theory of factors causing sex differences. At the time of fertilization of the egg cell, the sex chromosomes are the only factors that are different in XX and XY zygotes. Therefore, all sex differences result eventually from this genetic inequality. The expression of *Sry* causes differentiation of testes in males, whereas in the absence of *Sry* the autosomal or X genes cause differentiation of ovaries in females. Gonadal differentiation sets up lifelong differences in levels of gonadal hormones, which act to cause sex differences permanently (organizational actions) or reversibly (activational effects). The unequal sex chromosomes lead to sex differences in the expression of X and Y genes in nongonadal cells, which contribute to sex differences in cellular function (Based on Arnold, *Horm Behav* 2009, 55:570–8)

idea that all sex differences stem from the inherent imbalance in the sex chromosomes in the zygote (Fig. 5). These are the only factors that we know differ consistently between XX and XY zygotes. Therefore, all sex differences in the lifetime of the individual must derive from them. The XX versus XY difference includes several classes of factors. One class comprises the Y genes, found only in XY cells. A second class stems from the different number of X chromosomes. XX cells have two alleles of each X gene, compared to a single copy in XY cells. XX cells have X alleles with a parental imprint (a mark that influences expression depending on the parent of origin) from both parents, in contrast to an exclusive maternal imprint on X genes in XY cells. Any of these sex chromosome differences can lead to differences in gene expression in XX versus XY cells or animals, to cause sex differences.

The unequal effects of sex chromosome genes occur in any cell type in the body, but are most important in the embryonic cells that differentiate into gonads. There, cells with a Y chromosome and the *Sry* gene will differentiate into testes, whereas cells without *Sry* will differentiate into ovaries. Therefore, *Sry* expression, exclusively in males, sets up lifelong sex differences in sex hormones (androgens, estrogens, and progestins) secreted by the gonads. These hormones are the dominant factors causing sex differences throughout the body. Thus, sex differences in hormonal effects are downstream of sex chromosome effects in the gonads. Hormones from the testes are the primary organizational factors, causing males to develop differently from females. In adulthood, however, activational hormone effects contribute to sexual differentiation: ovarian hormones make females different from males, and testicular hormones make males different from females.

Outside of the gonads, sex chromosome effects also cause sex differences in liver cells, fat cells, brain cells, etc. The sex chromosome effects interact with sex hormone effects in the same cells. Although this interaction has not been studied much, we expect that sometimes the two will be synergistic. Thus, two male factors, for example, testosterone and Y genes, might act in XY cells to make them different from XX cells. In other cases, the two factors might be antagonistic or *compensating*. One example of an antagonistic effect was discussed above. Male hormones increase body weight, but male sex chromosomes decrease body weight. These antagonistic effects may be the result of convergent effects of the two factors within the same cells. Alternatively, in complex phenotypes such as body weight, the interaction might occur because testosterone acts on some cells to increase body weight, but a single dose of X genes acts at other sites to decrease body weight, relative to XX females.

Revisiting the Notion of the Female Default

The classic (mid-twentieth century) idea of sexual differentiation was that the female phenotype occurred in the absence of any sex-biasing signals. Thus, mice with one or two X chromosomes are females (with vagina and uterus) in the absence of the Y chromosome. The Y chromosome contains a major factor, *Sry*, that causes the male to deviate from the default female form. Extending the idea into hormonal theory, the female development of genitals and brain occurs in the absence of any sex hormones. The male hormone testosterone causes differentiation away from the female default. In general, the idea of the female default form suggested that females do not have any sex-biasing molecules that make them differentiate from the male form or from a neutral intermediate form.

Several observations undermine this simple theory. (1) Having two X chromosomes makes females different from males. A double dose of some X genes is quintessentially female and leads to female phenotypes. (2) Although ovarian secretions are not required for the formation of vaginal labia and a uterus, they are quite important as activational hormones causing females to deviate from males. Female patterns of behavior such as lordosis do not occur in the absence of ovarian

hormones. Thus, the female state requires ovarian hormones. (3) Recent studies demonstrate that female mice that develop in the absence of estrogens (because of knock out of the aromatase gene required for estrogen synthesis) are less feminine in their brain and behavior than females that have estrogens. This difference is found even when estrogens are replaced in adulthood, eliminating the idea that estrogens are merely playing a role as activational factors. Because treating female rodents during development with estrogens has long been known to make their brains and behavior more masculine, it is paradoxical that removing estrogens in aromatase knockout mice makes them more feminine rather than less feminine. In a simple theory, if estrogens are higher in one sex (e.g., as a metabolite of testosterone in males), they cannot be responsible for both masculinizing that sex and feminizing it. The resolution of this paradox is likely that estrogens act normally during some phases of development, at some sites of action, to cause masculinization of males, but at other phases and at different sites to cause feminization of females. Thus, both males and females have sex-biasing factors that make them different from the other. The female is not default.

The Neuroscience and Molecular Cell Biology of Sex Differences

The above discussion has focused on what can be called the first agents in a molecular cascade of sexual differentiation. The essential primary signals – estrogens and androgens and their respective receptors, and genes on the X or Y chromosome – initiate complex and region-specific signal transduction pathways that permanently alter brain development. Identifying and characterizing the downstream effectors of specific anatomical, biochemical, and physiological variables that differ in males and females is essential to the dual long-term goals of a complete understanding of sexual differentiation as well as identification of sources of vulnerability and/or protection against insults and disease in each sex. In-depth measurement of narrowly defined specific traits has provided novel insights and even surprises as to how the brain develops differently in males and females.

Regulation of Cell Number is One Mechanism of Brain Sexual Differentiation

The first and most prominent sex differences reported in the brain were in the size of particular regions, beginning with the song control nuclei of the zebra finch and canary and extending to at least three regions of the rodent nervous system, the SDN, AVPV, and SNB, as discussed above. When considering how a brain region can come to be different in size in one sex versus the other, there are a limited number of clear options: (1) more cells are born, (2) more cells die, (3) the cells are more tightly packed or larger, or (4) more cells migrate into a particular region in one sex versus the other. Fortunately, testing the first three options is relatively straight forward. Differences in cell size and packing were quickly dispensed as not explaining how

the SDN, AVPV, or SNB could be so different in size in males versus females. The most obvious explanation would seem to be that gonadal steroids in the male brain would stimulate cell proliferation, but instead it was independently discovered in all three regions that males and females begin life with the same number of cells, but they selectively die off in one sex versus the other. In the case of the SDN and SNB, more cells die in females. The opposite is true in the AVPV, where more cells die in males, resulting in a smaller region overall. Even more surprisingly, the cellular mechanisms regulating cell death and survival in each region are independently regulated by distinct signal transduction pathways. Ironically the SDN, probably the most intensely studied sex difference in the brain, is the poorest understood in terms of how cell death is regulated. What is known is also surprising in that it does not involve the classic cell death pathway genes *Bcl2* and *BAX*, which do regulate sex differences in cell death in another brain region. The most current thinking involves the potential of cell suicide induced by over excitation in females which is prevented in males by a calcium-binding protein called calbindin. The AVPV is the most complex of the regions in that there are two different neuronal types, dopamine neurons and GABA neurons, and they die in different ways. The dopamine neurons undergo classic apoptosis, also called naturally occurring cell death, and this is induced by estrogen in males. The GABA neurons, however, die because they are deprived of a survival factor which is downregulated by estrogen. Neither of these cell pathways are active in SDN neurons. The SNB is still further unique from the others in requiring the direct actions of androgens, not estrogens, and also involving a growth factor that is produced by the muscles that are innervated by the SNB (these are motor neurons). Rather than acting solely on the SNB neurons themselves, the androgens also act on the muscles of the penis to assure appropriate innervation and the survival of these essential motor neurons.

The consistent observation that specific subregions of the brain vary by size because of differences in cell death, not cell birth, led to a general lack of consideration of cell proliferation as a sexually dimorphic variable. This changed following the emerging realization that the adult hippocampus continues to make new neurons throughout life, prompting an examination of the rate of neurogenesis in the hippocampus of newborn male and female rodents. During a limited period early in life, the male hippocampus makes up to twice as many new neurons as the female, and neurogenesis in females can be stimulated to male levels by treating with estrogen, like many other masculinized traits. However, the male hippocampus is not twice as big, suggesting some compensatory cell death is also occurring. The functional significance of this restricted but robust period of sexually dimorphic neurogenesis is not yet known but may relate to sex differences in stress responding and spatial learning strategies later in life.

Rates of cell genesis are also sexually dimorphic in the developing amygdala, a brain region critical to social behaviors, fear and anxiety. In contrast to the hippocampus, the rates of cell genesis are higher in females than in males, and at least some portion of the newly born cells will become astrocytes instead of neurons. The lower rate of cell genesis in males is associated with higher levels of endocannabinoids, the brain's own marijuana. Treatment of females, with drugs

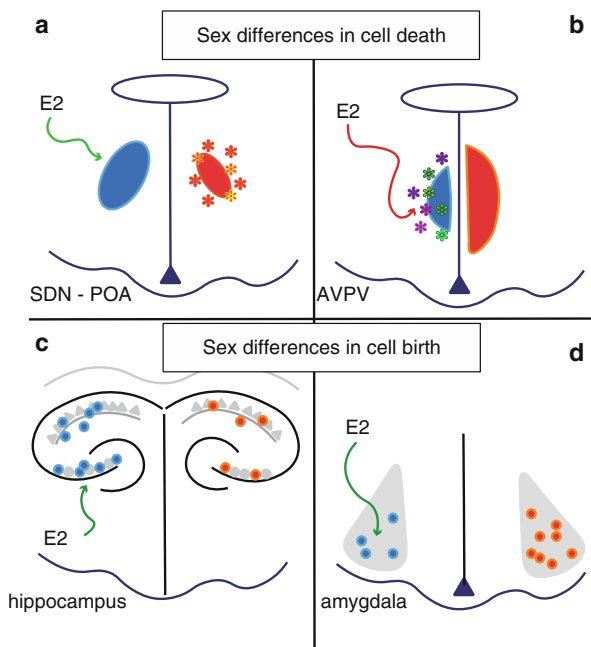


Fig. 6 Regulation of cell number is a mechanism of sexual differentiation. The size or composition of a brain region can vary between the sexes because of hormonal regulation of either cell death or cell birth. Here are examples of each. **(a)** SDN-POA; estradiol promotes survival of newborn neurons in the male. Because females do not have testicular androgens, and therefore much less estradiol in the brain, the cells undergo naturally occurring cell death, also called apoptosis. Dying cells are shown as asterisks. **(b)** AVPV; the opposite occurs in this brain region where higher estradiol in the male brain actually induces cells to die. Two different cell types, dopamine neurons and GABA neurons, undergo estradiol-induced cell death that is mediated by distinct mechanisms (details in text). **(c)** Hippocampus; here estradiol promotes cell birth (shown as *blue circles* in males and *red circles* in females) so that males have up to twice as many new neurons born during the early postnatal sensitive period. **(d)** Amygdala; here too there is a sex difference in the rates of cell proliferation, but this is because estradiol suppresses cell genesis in males, allowing for greater proliferation in females. *Red* and *blue circles* show newly born cells during the critical period, *red* for female and *blue* for male

that mimic marijuana and thereby activate endocannabinoid receptors, reduces the rate of cell genesis to that of males. In this case, the change in numbers of new born cells is correlated with sex differences in juvenile rough-and-tumble play, a trait more frequently exhibited by males of many species, including humans. An interesting consistency, between those brain regions that exhibit sexually dimorphic cell death and those that instead exhibit sex differences in cell birth, is the functional roles they are associated with. The SDN, AVPV, and SNB are all directly relevant to reproductive behavior or physiology, while the hippocampus and amygdala are components of an emotional regulatory circuit in the brain that modulates and integrates responses to stress, anxiety, and social cues (Fig. 6).

Synapse Formation and Elimination Builds Sexually Differentiated Circuits

Cell number is important as it forms the basis of the size of axonal projections and the number of targets for receiving synaptic input from other brain regions. But the number of synapses on an individual neuron can also vary, and this impacts the excitability of a particular cell. For example, a neuron of the preoptic area, a major brain region controlling male sexual behavior and female maternal behavior, receives synaptic input from the amygdala, a major brain region integrating olfactory information, fear, and social interactions. If a preoptic area neuron has on average twice as many excitatory synapses in one sex versus the other, a given external stimulus, such as the odor of a predator or a potential mate, would be predicted to have a much greater impact on the excitation of neurons in the sex with more synapses. This is indeed the case for the preoptic area where male neurons have twice the density of synaptic inputs as females. The sex difference in synapse density is organized by steroids during the critical period for sexual differentiation.

The close association of the preoptic area with adult male copulatory behavior provides an ideal system for in-depth interrogation of the signaling molecules by which the sexually dimorphic synaptic patterning is established. The goal has been to understand how steroids establish this increased number of synapses in this particular brain region. Given that the endpoint under investigation is a synapse, the obvious candidate would be a neurotransmitter of some sort. But surprisingly, the primary target for steroid regulation is not a neurotransmitter but a membrane-derived signaling molecule called prostaglandin E2 (PGE2). This is a short-lived, fast-acting signaling molecule normally associated with inflammation and fever, but more recently is recognized as a modulator of neuronal activity. In the preoptic area of developing males, estrogens derived from testicular testosterone induce the synthesis of PGE2 by stimulating the necessary synthetic enzymes. The increased PGE2 then initiates a series of events that involves at least two other cell types, the astrocytes and the microglia, which also contribute additional PGE2 (Fig. 7a). Microglia are particularly interesting as they are cells derived from the immune system, not the nervous system, but they reside within the brain. As with PGE2, microglia were previously thought to be important only following inflammation or injury, but are becoming increasingly appreciated for their role in normal brain development. Microglia produce PGE2, and this leads to increased production of glutamate receptors that are found at excitatory synapses, so that ultimately a neurotransmitter is critically involved. Nevertheless, the essential first step is from an unexpected source, prostaglandins.

Other discrete brain regions also have sex differences in synaptic pattern as well as dimorphic degrees of dendritic branching or length. While we do not have as deep an understanding of the molecules that mediate these sex dimorphisms as we do for the preoptic area, it is apparent that the molecules are different in each brain region and often involve more than just neurons (i.e., astrocytes and microglia). Thus, cell-to-cell signaling is an important part of constructing a sexually dimorphic circuit. In addition to acting locally within a discrete brain region, the signaling molecules may

also act to attract innervation by another region. This is evident in the circuit which controls gonadotropin secretion which is markedly different in males and females. The AVPV, which we discussed before, is larger in females. This is important because these neurons innervate and stimulate gonadotrophin-releasing hormone (GnRH) neurons, which induce luteinizing hormone (LH) release from the pituitary to initiate ovulation from the ovary. But the AVPV is also innervated by inhibitory neurons in a nearby region called the BNST (bed nucleus of the stria terminalis). In males, the number of neurons that send inhibitory projections to the AVPV is 10-times greater than in females. Recall that the AVPV is already smaller in males, and thus the large ratio of inhibitory input to a smaller number of neurons assures that the circuit in males is strongly inhibited and will not function in the same way it does in females. The initial construction of this sexually dimorphic circuit requires the release of attractant molecules from the AVPV that induce the axons of the BNST neurons to innervate them. The amount of attractant produced by the AVPV neurons is increased by estrogens and hence greater in males (Fig. 7b). Thus, in this circuit, estrogen plays the dual role of killing off the AVPV neurons while also assuring that those that remain are more heavily innervated by inhibitory BNST neurons, a remarkably coordinated but multifaceted effect of a single hormone.

Hormones Cause Epigenetic Changes as Part of Sexual Differentiation

When theories of sexual differentiation were first being developed, our understanding of the brain was far less sophisticated than it is today. The use of the term “organization” was accompanied by equally construction-oriented words such as the “neuronal architecture,” “blue prints,” and “wiring.” The general view was that the brain was built early in development, after which it remained unchanged. But we now know that the brain is far more malleable throughout life than we first thought.



Fig. 7 (continued) coordinated action of at least three cell types. Estradiol (E2) promotes the synthesis of the prostaglandin PGE2 in neurons by activating transcription of the synthetic enzyme, cyclooxygenase (COX). The PGE2 is released from the neuron and stimulates neighboring microglia to produce still more PGE2. Astrocytes release glutamate when stimulated by PGE2, and this glutamate binds to AMPA receptors which then promote the formation of excitatory spine synapses. **(b)** The AVPV is a critical brain region for activating GnRH neurons which in turn induce the release of the gonadotropin LH from the pituitary. LH is required for ovulation in females and is released in a highly coordinated surge into the blood stream at a specific time during the reproductive cycle. Neurons located in the AVPV provide excitatory input onto the GnRH neurons in order to induce the LH surge. Sex differences in AVPV output are produced during the sensitive period for sexual differentiation by two effects: estradiol-induced increase in the inhibitory input from the BNST to the AVPV and estradiol-induced reduction in the number of AVPV neurons. As a result, males do not release sufficient LH from the pituitary to generate the surge levels needed in the bloodstream to induce ovulation. Thus, a female brain function, not needed in males, is shut off. Together these examples illustrate how sexually dimorphic neural circuits are constructed

New neurons continue to be born in some regions, dendrites and axons grow and retract, and synapses come and go. In light of this new knowledge, we also have had to re-examine our thinking about the permanency of the organizational effects of gonadal steroids on the developing brain.

Epigenetics literally means “above the genome” and refers to modifications to the genetic material that do not involve changes in the DNA base-pair composition. The genetic code itself is not changed. Rather, epigenetic changes come in a variety of forms, but the two most common involve the addition of a methyl group to the cytosine nucleotides and the addition of an acetyl group to the histones, which are proteins around which the DNA is wrapped. Both of these changes can last a long time and impact whether or not a particular gene is transcribed.

Epigenetic changes to the genome can also occur in response to external stimuli such as stress or starvation, as well as in response to internal cues like hormones. It was this realization that led several research groups to simultaneously investigate whether the organizational actions of steroids during brain sexual differentiation were maintained by epigenetic modifications. There are two ways to approach this question: (1) look for evidence of epigenetic marks that are different in males and females and modified by hormones or (2) manipulate existing epigenetic changes and determine the impact on sexual differentiation. Both approaches have proven informative. In the preoptic area, there are sex differences in epigenetic marks of both types, with increased methylation of the DNA in females which is reduced by estrogen treatment (i.e., masculinization) and increased acetylation of key histones in males which is increased by estrogen treatment. One of the genes found to have more methylated DNA in females was the gene coding for the aromatase enzyme. This same gene was also found to have increased acetylation of the associated histones in males compared to females. The net result is increased expression of the aromatase gene in the preoptic area of males. Recall that this enzyme converts testosterone into estrogen, and thus the epigenetic changes in males are in essence a mechanism for positive feedback to assure that high levels of estrogen are produced locally in the preoptic area. Many other genes are modified epigenetically as well, and interestingly some of the epigenetic changes do not appear until long after the sensitive period for sexual differentiation. Scientists are still trying to understand how these changes occur and what the long-term impact is for the brains of males and females across the lifespan. One important message, though, is that whereas epigenetic changes are enduring, they are not necessarily permanent. Under certain conditions and in response to extreme stimuli, epigenetic changes can be reversed or new ones induced during an individual's life, unlike the DNA sequence of the genome.

The Brain is a Sexually Differentiated Mosaic

A recurring theme that should now be apparent is that when considering the molecular mechanisms that mediate the sexual differentiation of a particular brain region, what is true for one area is not true for another. The same holds for sexually differentiated behaviors and physiology. Some behaviors are largely determined by

early hormone exposure, while others are impacted by the sex chromosomes, and some are a combination of both. At first this seems to be a maddening level of complexity, but the bigger picture is the realization that nature has designed a system to assure that no two brains are exactly alike, even in our genetically homogeneous rodent strains. By invoking unique signaling systems in each region or for each behavior, it is essentially assured that there will be variability between them as there are so many distinct nodes for modification by internal and external factors. For example, in the preoptic area alone, we know there is one distinct set of signaling molecules that determine whether the neurons of the SDN will live or die, while another set will determine the number of excitatory synapses. In the case of the synapses, this involves multiple enzymes, kinases, receptors, and structural proteins, each one of which is subject to allelic variation and modification by cellular factors which can impact the final outcome. This same scenario holds true for every other region and its associated behaviors, which are controlled by multinodal circuits, further increasing complexity. These observations compel us to acknowledge that there is not a uniform “male brain” or “female brain” but that instead each male and female has a unique constellation of degrees of maleness or femaleness for each brain region or circuit, resulting in a brain mosaic. In some senses, this is intuitively obvious, as we all know that men and women vary enormously in their interests and behaviors both between and within the sexes (Fig. 8). What we do not know is how much biology versus cultural expectations and experience influence discrete brain regions in boys and girls or men and women to provide so much diversity both within and between the sexes.

Evidence That the Human Brain is Sexually Differentiated is Debated

The concept of the human brain as a gender mosaic is just one piece of a larger argument regarding whether there are any biologically based predictable sex differences in human brain and behavior. The impact of environment and experience on humans begins early and is pervasive. Biological males and females often develop in profoundly different social environments. It is therefore impossible to separate the effects of sex-biasing factors that originate external to the individual from those that are intrinsic, such as hormones or genes. Many studies using either histological analyses of postmortem brains or MRI images of living brains have detected sex differences in the size or shape of particular brain regions or structures. But adult humans have spent a lifetime living as men or women with all of the associated gender influences, many of which can impact how the brain develops and functions. For example, a woman who has spent her life as a caretaker, either taking care of her own or others’ children, working as a nurse, or a kindergarten teacher, etc., has had very different daily experiences than a man who has worked every day in construction or as an engineer. Experience has powerful influences on the brain, and so these gendered lifestyles might alter brain characteristics. It is a bit of a chicken-and-egg problem, do women become caretakers because they have female brains, or does society push girls to become caretakers and this then changes their brains? One way

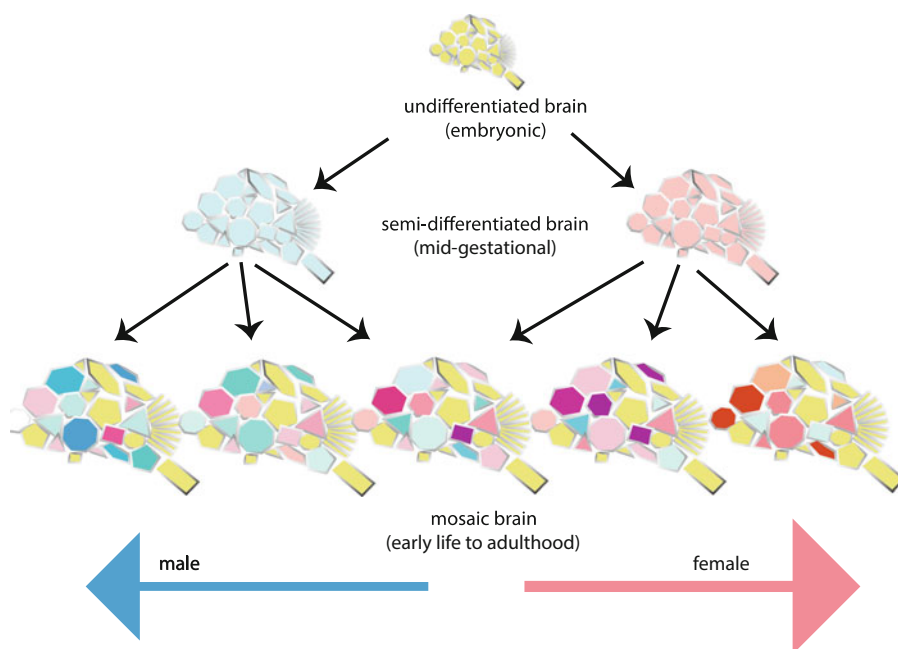


Fig. 8 The brain is a sexually differentiated mosaic. The brain is undifferentiated very early in gestation, but in females every cell is XX and in males every cell is XY which can begin to impact sex differences prior to the late gestational surge in hormones in males. Differences in the brain are broadly distributed, being found in regions relevant to reproduction, cognition, pain, anxiety, stress, and social behavior. But different mechanisms establish these sex differences in each case. Hormonal, genetic, and environmental factors contribute differently in different regions. The specific molecules that direct the establishment and maintenance of each sex difference also vary. Some regions do not vary at all between males and females. As a result, there is not a uniformly male or female brain (as there would be if there was only one mechanism operating in all regions). This scenario greatly increases variability within each individual and within each sex, generating a view of the brain that is a mosaic of relative maleness and femaleness

to avoid this conundrum is to study young children which, while certainly not free of the effects of gender expectations, at least have had less time to be influenced. Another approach is to use so-called natural experiments in which hormonal profile does not align completely with genetic or gonadal profile. One investigator, Melissa Hines, has combined both approaches by studying toy choice in young children. Her studies include girls who were exposed to androgens in utero due to a genetic anomaly. She finds that on average there is a clear bias in boys towards certain toys, i.e., guns, trucks, and gadgets, while girls more often prefer tea sets, doll houses, etc. But in the girls that were exposed as fetuses to androgens, toy preference is shifted towards that of boys, despite their having many other attributes typical of females. One insight into how this occurs is the recent observation that girls show a strong predilection to model the behavior of other girls and women, thereby reinforcing feminine characteristics. But this predilection was absent or only weakly

present in girls exposed to androgens prenatally. So it may not be that the androgens are impacting some brain region that determines a preference for playing with trucks. Rather, the impact may be on how the girls model their behavior on that of others. This example of a nature-versus-nurture convergence suggests that sexual differentiation of the human brain is complex and nuanced and that there is much more to be learned.

Sex Differences in Diseases of the Brain

Diseases of the brain can be loosely divided into those that are psychiatric or neurodegenerative. Neuropsychiatric disorders can be further subdivided into those with origins in development versus adult onset. Developmental disorders include autism spectrum disorders, attention deficit and hyperactivity disorders, and early onset schizophrenia. Disorders that mostly emerge only after puberty, and for which no clear developmental source has been identified, include major depressive disorders, anxiety and panic disorders, anorexia nervosa and bulimia, and bipolar disorder and late-onset schizophrenia. Neurodegenerative disorders include but are not limited to Alzheimer's disease, Parkinson's disease, Amyotrophic Lateral Sclerosis (Lou Gehrig's disease), multiple sclerosis, and Huntington's disease. Dyslexia and other learning or language impairments are not considered brain diseases but are nevertheless conditions that can cause distress. Every condition mentioned above, without exception, has been found to vary either in frequency, severity, or presentation in boys and girls and in men and women. The magnitude of the gender difference varies from extreme (anorexia is 14 times more likely in girls, attention deficit is 10 times more likely in boys), to modest (Parkinson's has a somewhat later age onset in women) to slight (Huntington's is marginally but significantly more severe in women). Each disease has its own genetic, environmental, or unknown origins. Understanding how and why the disease differs in males and females provides a unique wedge point to probe the biological origins and thereby identify previously undetected nodes for pharmacological intervention or prevention.

Understanding the factors that increase disease in one sex versus the other is not as easy as it sounds. Adult onset diseases are confounded by the fact that a lifetime of experience has preceded the diagnosis and men and women usually lead very different lives. For instance, do men suffer from Parkinson's more because they are exposed to an environmental toxin more than women? Do women suffer from depression more not because they are more depressed but because they are more willing to seek the assistance of a physician, whereas depressed men are more likely to seek solace in drugs or alcohol? Although depression is thought to be influenced by sex differences in biological factors, nonbiological factors also likely play a quite significant role.

The impact of nonbiological factors is reduced in younger subjects. For this reason, sex differences in developmental neuropsychiatric disorders have been of particular interest. Of the developmental neuropsychiatric disorders, none is more striking than the five times higher rate of diagnosis of autism spectrum disorders in

boys compared to girls. As noted at the beginning of this chapter, this suggests there is a protective factor in girls, and identifying that would be of benefit to both sexes. Autism is a genetic disease as it tends to run in families and is more frequent in identical than in nonidentical twins. But the genes that drive the disease have turned out not to be simple, with over 100 separate genes identified to date and most of them explaining less than 1 % of cases of autism. Nonetheless, there is evidence that females require a higher genetic “hit” or higher rate of mutation in order to be diagnosed with autism, confirming the notion of a protective effect in girls. However, there may also be a factor that makes males more vulnerable. The obvious culprit would be testosterone, and this has led to ideas such as the extreme male brain theory of autism. But there has been no clear relationship established between developmental testosterone levels and the risk of autism, suggesting there are far more complex factors at play. The same caveat likely holds true for all other diseases, but the profound impact of gender compels us to identify the sources of the variance between males and females in both health and disease.

Evidence suggests that numerous sex-biasing factors contribute to sex differences in multiple sclerosis (MS). Although MS occurs about 3–4 times more often in women, it often progresses more rapidly or severely in men. Thus, the disease pattern is difficult to explain based on a single protective factor in one sex. MS is an autoimmune disease in that the immune system abnormally attacks the myelin sheath surrounding neuronal axons. Sex differences in disease processes have been extensively studied in a mouse model of MS called experimental autoimmune encephalomyelitis (EAE). EAE is induced in mice by injecting them with myelin proteins plus an adjuvant that stimulates the immune system, which then attacks the mouse’s own myelin. As in MS in humans, female mice are affected more than males by EAE. The severity of EAE can be reduced in females by treating them with testosterone. It can also be reduced by treating them with estriol, an estrogen that is elevated during pregnancy in humans. The protective effect of estriol in mice is thought to mimic the reduction in symptoms of MS that occur naturally in pregnant women. Because of the effectiveness of testosterone and estriol at reducing the symptoms of EAE in mice, these hormones are being tested as attractive candidate drugs that might be used to alleviate MS in humans. Hormones are not the only protective factors in EAE, however. Sex chromosomes also play a role. The Y chromosome appears to be protective, and the presence of a second X chromosome appears to make EAE worse. Thus, XX mice show worse EAE than XY mice, independent of the type of gonad they have. Even more complicated is the finding that having an XY nervous system makes the reaction of the brain to EAE worse than having an XX brain. Thus, the immune attack on the brain may be worse in XX than in XY, but the response of the brain to the attack may be worse in XY than in XX. The sex differences therefore can push in opposite directions, offsetting each other, in much the same way as hormones and sex chromosome genes compensate for each other in studies of body weight and obesity discussed above. The result is a complex pattern of sex difference in EAE, with one sex being protected more than the other depending on which of the sex-biasing factors predominates.

Summary and Outlook

Historically, the study of sex differences satisfied the goal of understanding the processes controlling the divergent development of reproductive tissues that are strikingly different in females and males. These studies gave rise to a general theory of sexual differentiation. The experimental tests of the theory uncovered molecules that are inherently different in the two sexes, which cause sex differences in any tissue. More recently, increasing attention has been paid to the idea that many normal physiological and behavioral processes are sexually dimorphic. The incidence and progression of diseases also often differs in the two sexes. The classical theories of sexual differentiation are now being applied and tested to establish the molecules that account for sex differences in physiology and disease of many nonreproductive brain regions and organs. At the same time, the study of brain sexual differentiation is being advanced at the molecular, cellular, and circuit level to understand better which neurons and glia are affected, how these cells signal to each other differently in the two sexes, and how the sex-biased cellular functions give rise to coordinated sexually differentiated behaviors and functions. The recent explosion of interest in epigenetic factors is rapidly changing our models of sexual differentiation. Hormones and sex chromosome genes cause epigenetic changes in the genome, which are poorly understood but will be clarified in the near future. Particularly exciting is the idea that environmental factors, including those that are sex-biased, cause short-term or long-lasting epigenetic changes in the readout of the genome. Although we have long recognized that sex differences are caused in part because of gendered environments, biology has focused more on inherent biological factors rather than information coming from the environment. Because we now begin to understand physical epigenetic mechanisms by which environment interacts with the inherent biological signals, there exists the possibility of greater appreciation of the complex biological-environmental interactions that give rise to sex differences in physiology and disease.

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