

Two Perspectives on the Origin of Sex Differences in the Brain

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ABSTRACT: Most sex differences in brain function are attributed to sex differences in the effects of gonadal secretions. In addition, however, male and female cells differ because of differential effects of sex chromosome genes expressed within the cells themselves. The latter conclusion comes from numerous studies in which sexual phenotype appears to be insensitive to the effects of sex hormones during development or cases in which sex differences develop before the onset of sex-specific patterns of gonadal secretions. Recently, mouse models have become available in which the genetic sex of brain cells is independent of the gonadal type (testes vs. ovaries), which allows a test of the role of sex chromosome genes in brain development. This paper reviews the evidence that genetic sex of brain cells influences their sexual phenotype, and critically discusses the relative advantages of various experimental approaches to study this effect.

KEYWORDS: sex chromosomes; sexual differentiation; Y chromosome; X chromosome; songbird; genetic models

TWO VIEWS OF SEXUAL DIFFERENTIATION

Almost a century ago, Lillie¹ and Keller and Tandler² published observations on freemartin cattle, suggesting strongly that the sexual phenotype of reproductive tissues develops under control of endocrine secretions carried in the blood. Female freemartin calves share their fetal blood supply with a male twin, resulting in masculinization of some of their reproductive tissues (genitals, gonads, etc.). The humoral masculinizing factors, they concluded, derived from the male. The same factors likely were responsible for determining the masculine phenotype of the normal male's own reproductive organs. These ideas were conclusively supported by the classic experiments of Jost in the late 1940s,³ who manipulated gonadal secretions

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Ann. N.Y. Acad. Sci. 1007: 176–188 (2003). © 2003 New York Academy of Sciences.
doi: 10.1196/annals.1286.018

prenatally to prove that fetal secretion of testosterone from the testes was responsible for masculine (male-typical) differentiation of the genitalia (penis, scrotum) and Wolffian ducts. Jost also inferred the existence of a testicular Müllerian inhibiting hormone that causes involution of the Müllerian structures in males.⁴ In 1959, Young and colleagues suggested that testicular secretions also induce masculine differentiation of the brain because they found that female guinea pigs given testosterone during fetal life displayed more masculine behavior and less feminine behavior than normal females.⁵ These classic experiments, and many others performed subsequently, gave rise to the important conclusion that many tissues of the body are sexually differentiated permanently by the action of gonadal hormones, especially testosterone and its metabolite estradiol.⁶⁻⁸ The effects of gonadal hormones are impressive, and thus the study of sexual differentiation has appropriately focused on the mechanisms of hormonal action. Moreover, the dominant effects of gonadal hormones suggested that other factors need not be invoked to explain sexual differentiation. For example, because XX and XY individuals both develop a masculine phenotype if they are exposed to testicular secretions during fetal or neonatal periods of development, it appears that an XX or XY genotype is not a pervasive determinant of phenotypic sex.

In its extreme form, the dogma of gonadal origin of somatic sexual differentiation has a corollary, usually implicit, that XX and XY cells are functionally equivalent unless gonadal secretions act on them in a sex-specific fashion. This corollary conflicts with another perspective that emerged from studies of genes encoded on the sex chromosomes. Starting around the turn of the twentieth century, various investigators linked phenotypic sex to the sex chromosomes in several invertebrate organisms. By 1920, for example, the sex of *Drosophila* was found to be related to the number of X chromosomes, not the presence or absence of the Y chromosome, because XO flies are male.⁹ Similarly, a mechanism that measures X gene dosage determines sex in the nematode *Caenorhabditis elegans*.¹⁰ Not until 1959 was it established that the mammalian Y chromosome plays a dominant male-determining role (XO humans and mice have ovaries and XXY individuals have testes).¹¹ The testis-determining gene on the mammalian Y chromosome is *Sry*, which is necessary and sufficient to cause the formation of testes in mice.¹² The *Sry* gene is transiently expressed in the undifferentiated gonadal ridge of XY embryos, where it acts to initiate a complex cascade of molecular and cellular events that lead to formation of the testis. Thus, cell-autonomous sexual differentiation, caused by an unequal effect of sex chromosome genes, occurs in numerous unrelated animal species, either because of the male-specific action of Y genes or the difference in dose of X genes.

What features of the X and Y chromosomes influence our thinking about their potential to differentiate XX and XY cells? Could X gene dosage contribute to the difference in XX and XY cells in mammals, as in flies and *C. elegans*? In each of these species, mechanisms exist to reduce the effect of the sex difference in X gene dosage, presumably because the balance of X gene dose to autosomal gene dose is important for healthy functioning of individuals of both sexes. That balance would be disrupted in one sex if the X genes were expressed at a much higher level in females than in males. In mammals, dosage compensation is accomplished by X-inactivation, in which one of the two X chromosomes is transcriptionally silenced in each nongermline cell.¹³ X-inactivation is incomplete and imperfect, however, because some X genes escape inactivation,¹⁴ and thus XX cells probably are not equiv-

alent to XY cells in dosage of all X genes. The mammalian Y chromosome is small and heterochromatic.¹⁵ The human Y chromosome encodes only 27 different proteins.¹⁶ In mice, only 12 Y-linked genes have been identified to date. Eight Y genes are expressed in the brain of males and could have a male-specific effect on the brain (discussed further in Refs. 17 and 18). Finally, dosage of X and Y genes is not the only difference between XX and XY cells attributed to the sex chromosomes. XX cells contain an X chromosome that received a paternal genomic imprint, whereas XY cells do not. These differences in genomic imprinting could contribute to cell-autonomous differences in male and female cells.^{19,20}

This discussion highlights the inherent nonequivalence of XX and XY cells, at least in some tissues and at specific times of development. Accordingly, studies of the sex chromosomes and genetics of sex determination evoke a different attitude towards sexual differentiation of the brain than do studies coming from the endocrine tradition. Although no one doubts the strong effect of testosterone to cause masculine development of the brain and other tissues, one might wish to stop short of pronouncing XX and XY brain cells functionally equivalent. The question then becomes, how are XX and XY cells different, and does this difference have any effect on sex differences in phenotype of the brain? To discuss this question, we briefly review previous research suggesting that sex differences are caused by cell-autonomous action of sex chromosome genes in mammals and birds. These cases are grouped according to the experimental paradigms used, to illustrate the strengths and weaknesses of specific approaches.

Sex Differences in Phenotypes That Resist Sex Reversal by Manipulations of Gonadal Steroid Hormones

Zebra-finch males sing a courtship song that females cannot sing. Accordingly, the brain regions controlling courtship song are much larger in males than in females.²¹ Attempts to test the role of gonadal steroids in sexual differentiation have met with mixed results. Treating neonatal females with testosterone or estradiol causes significant masculinization of the brain circuit and as adults such females sing,²² suggesting that the male is normally masculinized by his own testicular secretions. Although numerous hormonal treatment regimes have been used in a variety of studies, the neural song circuit of steroid-masculinized females is only about half as masculine as that of a normal male (e.g., Refs. 23 and 24). Moreover, attempts to prevent masculine development in males, by blocking androgen or estrogen action *in vivo*, have often been unsuccessful²⁵ (but see Refs. 26 and 27). Similarly, the dramatic sex difference in plumage of zebra finches is not sex reversed by a wide variety of manipulations of gonadal steroids at various times of life. Although one is tempted to invoke other factors to explain sexual differentiation when steroid-induced sex reversal is incomplete,^{24,28} the lack of an hormonal effect is not strong evidence favoring cell-autonomous actions of sex chromosome genes.²⁵ In each case, the failure of the sex steroid treatment is potentially attributed to use of the wrong dose, wrong treatment period, or an ineffective drug, etc. Experiments that manipulate steroid hormones test a role for those hormones, but do not provide a strong test for the role of other factors.

In zebra finches, it has been possible to manipulate the phenotype of the gonad in genetic females to compare brain phenotype in females with ovaries and females

with testes. Because ovarian development in birds requires the action of estrogen at early stages of gonadal development, females are induced to develop testes if they are treated as embryos with an inhibitor of aromatase, the synthetic enzyme for estrogen. This treatment typically causes development of a testis on the right side of the animal and an ovotestis on the left side. Despite the presence of a large quantity of testicular tissue in some of these birds, the neural song circuit is masculinized little or not at all.²⁹ This result raises doubts about the importance of testicular secretions in the development of a masculine song circuit.

Sex Differences Detected prior to Gonadal Differentiation

In some cases, sex differences can be observed in nongonadal tissues prior to the time of gonadal differentiation and/or sex-specific gonadal secretions. For example, XY embryos grow faster than XX embryos in mice, rats, cattle, and humans^{30,31} even before gonads have developed. In mice, both the dosage of X gene(s) and male-specific action of Y gene(s) participate in this sex difference.^{32,33} In tamar wallabies, the scrotum begins to differentiate in XY males, and mammary tissue begins to differentiate in XX females, prior to gonadal differentiation.³⁴ X dosage is implicated because XXY wallabies have feminine phenotype in these tissues.

Reisert, Pilgrim, and colleagues have investigated the differences in the *in vitro* phenotype of XX and XY brain cells harvested from rat and mouse embryos, just after gonadal differentiation but prior to the stage at which large sex differences in plasma levels of steroid hormones have been detected.^{35,36} For example, when mesencephalic or diencephalic cells are harvested from day 14 rat embryos, then dissociated and grown under identical conditions *in vitro*, XX cultures differ from XY cultures in several respects, including the number of tyrosine hydroxylase immunoreactive neurons, the size or neuritic length of dopamine neurons, the effects of neurotoxin, or the number of prolactin immunoreactive neurons. These investigators conclude that the difference in XX and XY cells is not the result of the action of gonadal hormones, but rather is caused by cell-autonomous factors, presumably those on the sex chromosomes. This conclusion is also supported by recent experiments using a different experimental approach discussed below.

Studies of Genetic Anomalies in Which Genetically Male and Female Cells Are Exposed to a Common Gonadal Hormone Environment

It is experimentally difficult to compare XX and XY cells that are exposed to an identical hormonal environment in order to determine the role of the sex chromosomes in specific traits. Infrequently, nature performs this experiment for us, as in the case of bilateral gynandromorphs. Recently, Agate *et al.*³⁷ studied a gynandromorphic zebra finch in which the right half of the body was genetically male, and the left half genetically female. The plumage on the right half was typical of males, with an orange cheek patch, zebra stripes and a black bar on the breast, and white spots under the wing. On the left half of the body, the plumage lacked these characteristics and was gray, typical of females. The right gonad was a testis, and the left gonad an ovary. These findings suggest that the genetic mechanisms responsible for sexual differentiation of plumage and gonadal phenotype, both of which are thought to be caused by cell-autonomous action of sex chromosome genes, were lateralized with

female determinants on the left and male determinants on the right. In birds, the sex chromosomes of females are ZW and ZZ in males. Analysis of the gynandromorph's genomic DNA showed that W genes were present at a higher level on the left than on the right. Moreover, the expression of W genes was largely restricted to the left half of the brain, and a Z gene was expressed higher in the right brain, suggesting that the brain was genetically female on the left and male on the right. Because the two halves of the brain would have been exposed throughout life to the same levels of gonadal hormones, the sexual phenotype of the two sides of the brain would be expected to be the same if gonadal hormones were the only factors responsible for sexual differentiation. In contrast, if the genetic sex of cells in the brain contributes to sex differences in brain phenotype, then the genetically male right side of the brain would be expected to be more masculine than the genetically female left side. The neural circuit for song was found to be more masculine on the right than on the left, supporting the idea that sex chromosome genes act cell-autonomously to influence sexual differentiation. However, because both sides of the gynandromorph's brain were more masculine than those in females, diffusible factors such as gonadal hormones (or masculinizing hormones from the male half of the brain) apparently also played a role. The gynandromorphic male is reminiscent of a half-male, half-female wallaby.^{38,39} In this wallaby, the male side had a hemi-scrotum and the female side had a hemipouch, again confirming the cell-autonomous action of sex chromosome genes.

Behavioral Differences in Strains of Mice That Differ Only in the Allelic Composition of the Y Chromosome

Maxson compared aggressive behavior in congenic mouse strains that are genetically identical except for the alleles present on the Y chromosome.⁴⁰ DBA/1 males are more aggressive than C57BL/10 males when they are tested in encounters with males of the same strain. When the C57BL/10 Y chromosome is crossed onto a DBA/1 background (by crossing C57BL/10 males with DBA/1 females and backcrossing each successive generation of males to DBA/1 females), the resulting males (DBA1.C57BL10Y) differ genetically from DBA/1 males only in the non-pseudoautosomal region of their Y chromosomes. (The nonpseudoautosomal region does not recombine with the X chromosome and therefore contains genes specific to the Y chromosome.) DBA/1 males (with a DBA/1 Y chromosome) are more aggressive than DBA1.C57BL10Y males (with a C57BL/10 Y chromosome). Thus, allelic differences in the Y chromosome lead to differences among males in their aggressive behavior. Numerous other studies performed on different mouse strains and using different paradigms of testing indicate that the Y chromosome contains genes that influence aggression.⁴¹ If Y genes differ in their influence on aggression, it is logical to assume that the presence or absence of Y genes (when comparing males and females) might also contribute to sex differences in aggression.

These classic techniques of behavioral genetics demonstrate the Y linkage of a behavioral trait. They are designed to test the importance of different Y alleles, but are not designed to test the importance of gonadal hormones in sexual differentiation. For example, in the congenic mouse strains, Y genes could increase the secretion of testosterone by the gonads or adrenals, either at the time of testing of the gonadally intact males used in these studies, or during other times of life such as

perinatal critical periods of sexual differentiation.^{42,43} Measurements of plasma hormone levels suggest that strain differences in hormone levels may occur at specific ages.^{44,45} In general, however, even when no difference in the level of hormones is found, the evidence does not rule out differences in hormone levels at other ages.

Two strategies can be followed to attempt to resolve whether Y-linkage implies a cell-autonomous action of Y genes in the brain, or whether the Y effect is explained by an hormonal or some other effect. The best method is to determine which Y genes(s) is responsible for the effect, and to determine where in the brain or body the expression of the Y gene(s) impacts aggressive behavior. This could involve, for example, manipulation of Y gene expression in specific tissues such as the brain cells using transgenic or other methods. Another approach is to attempt to eliminate the hormonal differences between the congenic strains, to determine whether the Y effect persists in mice that have similar hormonal profiles. For example, one might test the aggressive behavior of animals that are gonadectomized and treated with equivalent levels of testosterone, to determine whether the Y effect is found when testosterone levels are eliminated as a potential contributing variable at the time of testing. Alternatively, perinatal animals may be treated equivalently with gonadal steroids or steroid hormone antagonists in an attempt to ensure that the strains receive the same gonadal steroid effects during development. Although such studies can help suggest whether steroids do or do not mediate the Y gene effect, they are not completely satisfactory because it is experimentally impossible to make two different strains hormonally equivalent.

***Comparison of Mice with Similar Gonads but Different Sex Chromosomes:
XX Males vs. XY Males and XX Females vs. XY Females***

To investigate the role for X and Y genes, one must vary the action of those genes and observe the effect on the phenotype. One particularly promising approach has been developed by Burgoyne and Lovell-Badge and colleagues.⁴⁶⁻⁴⁸ This involves the comparison of mice in which the complement of sex chromosomes (XX or XY) is made independent of the phenotype of the gonads. In these mice, a 12-kb region of the Y chromosome is deleted, removing the *Sry* gene. This "Y⁻" chromosome no longer causes differentiation of testes; thus, XY⁻ mice have ovaries and are female (defining sex by gonadal phenotype). Some mice carry an *Sry* transgene inserted into an autosome, so that XY⁻*Sry* mice are fully reproductive males. When one crosses XY⁻*Sry* males with XX females, four types of progeny are produced: XY⁻*Sry* males, XX*Sry* males, XY⁻ females, and XX females. These four genotypes allow a 2X2 comparison of mice with different gonadal phenotypes (testes vs. ovaries) and with different complements of sex chromosomes (XX vs. XY⁻). When one compares animals with the same gonadal type but with different sex chromosomes (XY⁻*Sry* males vs. XX*Sry* males, or XY⁻ females vs. XX females), differences between groups can be attributed to an effect of sex chromosome complement. When one compares animals with the same complement of sex chromosomes but different gonadal type (XY⁻*Sry* males vs. XY⁻ females, or XX*Sry* males vs. XX females), differences in phenotype can be attributed to an effect of the *Sry* transgene. Because the main known effect of *Sry* is to induce differentiation of the testes, the *Sry* effect is probably mediated mainly by differences in gonadal secretions. However, the *Sry* transgene is also expressed in nongonadal tissues such as the brain; thus, the hormonal

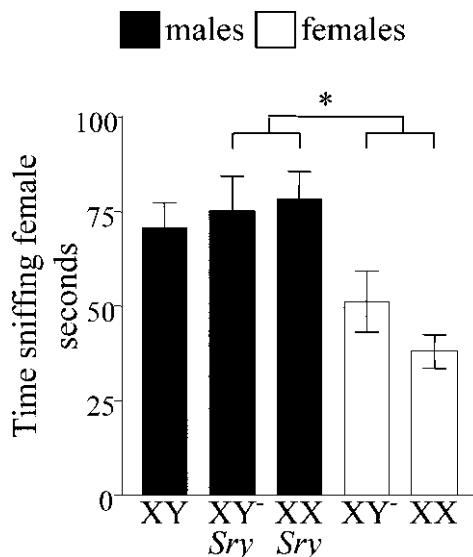


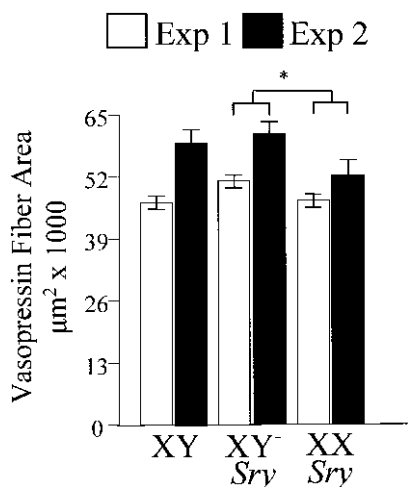
FIGURE 1. Sexual differentiation of social exploration. Five genotypes of mice were compared in a choice paradigm in which they could sniff an anesthetized male or female mouse in a 10-min social exploration test (means \pm SEM). The graph shows the amount of time spent sniffing the female. Males (black bars) spent more time sniffing the stimulus female than did females (clear bars, $P < 0.0005$), suggesting that gonadal secretions control sexual differentiation of this trait. The complement of sex chromosomes (XY⁻ vs. XX) did not have a significant effect. (Data from De Vries *et al.*⁴⁹)

differences between these groups are confounded potentially by *Sry* effects in the brain itself. Using the two-way ANOVA, one can test the separate effects of sex chromosome complement and gonadal type, and their interaction.

De Vries *et al.*⁴⁹ and Markham *et al.*⁵⁰ compared these four genotypes by measuring numerous brain structures and behaviors that were predicted or known to be sexually dimorphic in mice. These variables included measures of male copulatory behavior and social exploration, and structural traits in the lateral septum, anterior hypothalamus, cerebral cortex, and lumbar spinal cord. Prior to behavioral testing, all animals were gonadectomized and treated equally with Silastic capsules containing testosterone. By holding testosterone levels constant in between-group comparisons, any differences between groups could be attributed to factors other than levels of gonadal secretions at the time of testing (i.e., “activational” effects). On most measures, male mice (those with *Sry*) were different than female mice (those without *Sry*), independent of sex chromosome complement (FIG. 1). For example, males showed more masculine copulatory behavior than females, and their complement of sex chromosomes (XX vs. XY⁻) had no effect. Thus, for these variables gonadal secretions during development appear to be the primary determinant of sexual phenotype, as predicted by the strong differentiating role of testosterone in many previous studies in which sexual phenotype is reversed by altering testosterone or estradiol

action in the period around birth in rodents. In the lateral septum, males also had a higher density of vasopressin fibers than females, but, in addition, the complement of sex chromosomes had an effect (FIG. 2). Thus, $XY^{-}Sry$ males had a higher density of vasopressin fibers than $XXSry$ males, and when females from all female litters were compared, XY^{-} females had a higher density than XX females. The results indicate that the genetic sex of cells (XY^{-} vs. XX) influences this trait. When the complement of sex chromosomes is similar to that possessed by normal XY males, the trait is more masculine than when the complement of sex chromosomes is similar to that of normal XX females.

A: males



B: females

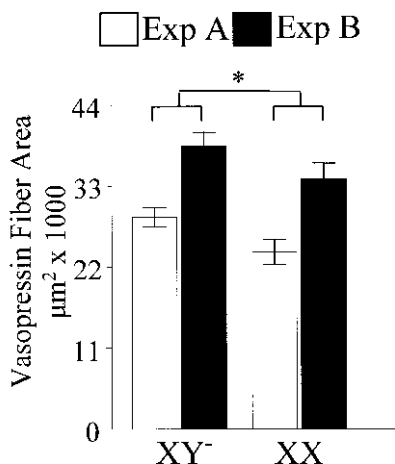


FIGURE 2. (A) The density of vasopressin immunoreactive fibers in the lateral septum of male genotypes (means \pm SEM). Black and clear bars represent the results from two different experiments. $XY^{-}Sry$ males had a higher density than did $XXSry$ males ($P < 0.05$), showing an effect of sex chromosome complement on this trait. (B) Vasopressin fiber density in females from all-female litters shows the same effect of sex chromosome complement because XY^{-} females had a higher density than XX females in two experiments ($P < 0.02$). (Data from De Vries *et al.*⁴⁹)

Does this finding prove that sex chromosome genes act within cells in the brain to contribute to sex differences in phenotype? If so, does it contradict the classic idea that sex differences in brain phenotype are caused by gonadal steroid hormones? The conservative answer is that the finding shows an effect of sex chromosome complement, but does not answer where or how the effect is mediated. It is possible that the action of X and Y genes is outside of the brain. For example, one may question whether XY^-Sry males and $XXSry$ males, or XX and XY^- females, are hormonally equivalent. In both cases, the gonads are functionally different. For example, $XXSry$ males lack Y genes necessary to make sperm; therefore their testes are smaller than those of XY^-Sry males. XY^- females are subfertile. Despite these apparent differences in functional capacity of the gonads, the two male groups are phenotypically similar in their brains and behavior, as are the two female groups. Furthermore, XX animals were probably not exposed to different levels of gonadal hormones than XY^- animals because such a difference should have influenced several of the brain phenotypes measured, which are all barometers of hormonal action. However, because the experiment primarily involved manipulation of the sex chromosomes, not manipulation of hormonal secretions, the results primarily bear on the role of X and Y genes. As discussed above, comparisons of animals with different genotypes are aimed at a strong answer to the question of which genes are involved, not at resolving the possibility of hormonal mediation.

How can one answer the interesting question of whether the sex chromosome effect is mediated by hormonal or other mechanisms? The best approach is to determine which X or Y genes are involved, and determine where and how they act to contribute to a phenotypic difference. If XX and XY^- animals differ in phenotype, then one can narrow down the genes responsible to the X or Y chromosome, for example, by comparing XO vs. XX (varying X dose independent of Y dose) and XO vs. XY (varying Y dose independent of X dose), as was done when measuring effects on mouse embryo size.^{32,33} If a specific gene can be implicated, then varying the dose of that gene is feasible, for example by transgenic insertion of Y genes into XX mice.⁴⁷ Once a specific gene is implicated, its sites of expression and mechanisms of action can be studied to determine whether it acts via a hormonal intermediate or via direct action on the brain.

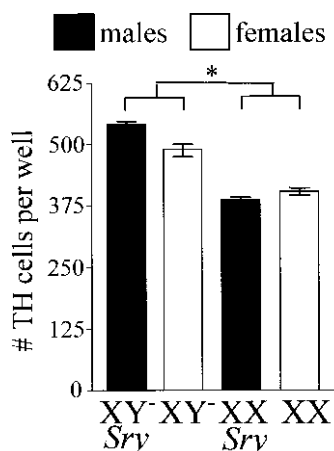


FIGURE 3. Effect of sex chromosome complement on neuronal phenotype *in vitro*. Mesencephalic cells were harvested from day 14 embryos, dissociated, and plated out. When measured 6 days later, cultures derived from XY^- embryos (either XY^-Sry males or XY^- females) contained more tyrosine hydroxylase immunoreactive neurons than did XX cultures (derived from $XXSry$ males or XX females) ($P < 0.00001$). The primary determinant of this sex difference *in vitro* is the complement of sex chromosomes. (Data from Carruth *et al.*⁵¹)

The four genotypes described above have also been used to study the sex difference in phenotype of XX and XY mesencephalic cells *in vitro*. Using the paradigm pioneered by Reisert and colleagues, Carruth *et al.*⁵¹ dissociated mesencephalic cells from mouse embryos on embryonic day 14. These cells were grown under identical conditions for 6 or 11 days before their phenotype was measured. The XY cultures, harvested from male (XY^{-Sry}) or female (XY⁻) embryos, developed more tyrosine hydroxylase immunoreactive neurons than XX cultures harvested from embryos of either sex (XX^{Sry} or XX) (FIG. 3). The sex chromosome effect was specific to TH-immunoreactive neurons, since the total number of neurons was not different between groups. Thus, for this phenotype *in vitro*, sex chromosome complement is the primary determinant of the differences observed, not any gonadal hormones that might circulate as early as embryonic day 14.

CONCLUSION

Because the field of neuroendocrinology has long focused on the dominant differentiating role of testosterone in sexual differentiation of the brain, based on abundant evidence supporting this role for many sexually dimorphic traits, there is understandable resistance to the view that the genetic sex of brain cells might also contribute to differences in XX and XY brains. Viewed from the perspective of sex chromosome geneticists, however, the equivalence of XX and XY brain cells is doubtful, but the evidence to date does not indicate how different these cells are, or how the difference interacts with testosterone's role in sexual differentiation. In part, the absence of information is the result of the paucity of experimental models in which XX and XY brains can be compared under conditions in which gonadal hormone levels are similar or equivalent. Fortunately, at least in mice, the ability to dissociate sex chromosome complement from gonadal phenotype now offers opportunities for further measurement of sex chromosome effects. The first results using these models suggest that the genetic sex of brain cells plays a role in sexual differentiation. Other mouse models may also be useful, for example, mice in which gonadal development is prevented by deletion of steroid factor 1 or other genes critical for gonadal development.⁵² In quail, the genetic sex of the brain has been altered recently by transplanting genetically male brain tissue into the body of a female host and vice versa.⁵³ Under these conditions a genetically female quail brain appears unable to sustain normal testicular development, adding support to the importance of the sex chromosomes in determining sexual phenotype. We are optimistic that future studies will lead to identification of specific sex-chromosome genes that impact brain development and behavior in a sex-specific fashion.

ACKNOWLEDGMENTS

We thank Paul Burgoyne, Robin Lovell-Badge, and Amanda Swain, who have taught us a great deal about the sex chromosomes and methods to investigate sex chromosome effects on neural and behavioral development. This work was supported by National Institutes of Health grants MH59268 and NS43196.

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