

Neural, not gonadal, origin of brain sex differences in a gynandromorphic finch

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In mammals and birds, sex differences in brain function and disease are thought to derive exclusively from sex differences in gonadal hormone secretions. For example, testosterone in male mammals acts during fetal and neonatal life to cause masculine neural development. However, male and female brain cells also differ in genetic sex; thus, sex chromosome genes acting within cells could contribute to sex differences in cell function. We analyzed the sexual phenotype of the brain of a rare gynandromorphic finch in which the right half of the brain was genetically male and the left half genetically female. The neural song circuit on the right had a more masculine phenotype than that on the left. Because both halves of the brain were exposed to a common gonadal hormone environment, the lateral differences indicate that the genetic sex of brain cells contributes to the process of sexual differentiation. Because both sides of the song circuit were more masculine than that of females, diffusible factors such as hormones of gonadal or neural origin also likely played a role in sexual differentiation.

Theories of sexual differentiation in birds and mammals postulate different mechanisms for sexual differentiation of gonadal and nongonadal tissues (1). Gonadal sex is determined by sex chromosome gene(s). In mammals, the Y-linked *SRY* gene is expressed within cells in the undifferentiated gonadal ridge to induce testicular development (2). In contrast, sexual differentiation of nongonadal (somatic) tissues, such as the brain, is caused by sex differences in early gonadal hormones (3, 4). A key question is whether somatic sexual differentiation also involves cell-autonomous action of sex chromosome genes as occurs in gonadal differentiation.

An exclusively hormonal theory of brain sexual differentiation has been challenged by studies of zebra finches (*Taeniopygia guttata*). Males, but not females, sing a courtship song. The male's neural song nuclei are much larger and have larger neurons (5). Treatment of hatchling females with estradiol induces more masculine neural development, and they sing (6), suggesting that estrogens derived from gonadal secretions normally induce masculine song system development in males. However, treatments with gonadal hormones do not completely sex-reverse females, and blocking testicular hormones in males does not prevent masculine development (1). Moreover, testicular tissue induced to develop in genetic females does not masculinize the song system (7). One explanation for these results is that sex chromosome genes are expressed differently within brain cells of the two sexes and act in a cell-autonomous fashion to cause differences in song system development (8).

The discovery of a rare bilateral gynandromorphic zebra finch presented us with an opportunity to test this hypothesis, because cells on one half of its brain and body were genetically male and cells on the other half were genetically female. Because both halves of the brain developed in the same gonadal hormonal environment, a completely gonadal hormone theory of sexual differentiation predicts that the sexual phenotype of the song circuit on both sides of the brain would be equally masculine or feminine. In contrast, if the genetic sex of the cells contributed

to sexual differentiation, then the genetically male half is predicted to be more masculine than the genetically female half. To test these ideas we measured the genetic sex and sexual phenotype on both sides of the brain and other body parts. The results suggest that sexual differentiation is controlled by both the cell's genetic sex and its hormonal environment.

Methods

Animals and Tissue Collection. Animal use was approved by the University of California, Los Angeles, Chancellor's Animal Research Committee. The gynandromorph was found in the colony of Fernando Nottebohm at The Rockefeller University, New York, and kindly given to us. We observed its reproductive behavior in the presence of a female at least weekly for 21 months. Tissue was collected after the bird was anesthetized with Equithesin and perfused intracardially with saline to remove as much blood as possible. Fresh brain (lateral telencephalon), muscle, and feathers were sampled on each side for analysis of genomic DNA. The brain was frozen-sectioned in a frontal series: three sections at 30 μm , three at 10 μm , and three at 5 μm , repeated throughout the brain. For measurements of volumes and neuronal cell sizes, 30- μm sections were fixed in 4% paraformaldehyde and stained with thionin. Other sections were processed for *in situ* hybridization to detect expression of mRNAs. Gonadal tissue was immersed in Bouin's fixative for 24 h, washed in 70% ethanol, paraffin-embedded, sectioned at 6 μm , and then stained with hematoxylin and eosin. The syringes (vocal organs) of the gynandromorph and one control male and female were freshly dissected and immersed in Bouin's fixative, paraffin-embedded, sectioned at 10 μm , and trichrome-stained.

Measurements of the brain were made in control birds derived from previous studies (9–11, ||). Most of the controls were untreated, but a few received blank Silastic pellets as controls. The controls were killed at 57–137 days and perfused with 0.75% saline, then 10% phosphate-buffered formalin. Brains were embedded, immersed in 20% sucrose in 10% phosphate-buffered formalin, frontal-sectioned at 30 or 40 μm , and stained with thionin.

Brain Measurements. In the gynandromorph, the volumes of HVC (high vocal center), RA (robust nucleus of the archistriatum), and Area X were determined by measuring the area of the nucleus in sections at intervals $\leq 135 \mu\text{m}$ throughout its rostro-caudal extent. The area in one section was multiplied by the

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Abbreviations: HVC, high vocal center; RA, robust nucleus of the archistriatum; AR, androgen receptor.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF539981, AF539982, and AF532914).

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distance between the leading edge of that section and the next. The sum of these volumes represented the volume of the nucleus. Song region volumes in controls were measured similarly, except the sampling interval was 90 or 120 μm for brains sectioned at 30 or 40 μm , respectively. The cross-sectional areas of 50 neurons per side of the gynandromorph (25 neurons per side in controls) were measured at $\times 800$, sampled throughout the rostrocaudal extent. All birds were measured blind to condition.

Genetic Analysis. The genetic sex of tissues on both sides was determined by genomic PCR and Southern blot analysis (12). Genomic PCR amplified fragments of the homologous sex chromosome genes *CHDIZ* and *CHDIW*. The primers amplify across an intron that discriminates between the two genes (*CHDIW* product 389 bp in ZW female cells, *CHDIZ* product 353 bp present in both ZW and ZZ cells). For Southern blots, genomic DNA was isolated, digested with *Bgl*II, and loaded at 5 μg per lane for muscle and 3 μg per lane for feather pulp. The blot was hybridized with a probe encoding the W gene *ASW*, which recognizes female-specific DNA bands. Approximately equal loading of lanes in the Southern blot was demonstrated by equivalent ethidium bromide staining of DNA (data not shown). The DNA content of blood cells was determined by suspending cells in buffer, staining DNA with propidium iodide, and measuring amount of fluorescence per cell in a flow cytometer by using the method of Krishan (13).

In Situ Hybridization. ^{33}P -labeled antisense riboprobes were hybridized to tissue sections as described (12) to detect mRNA expression of androgen receptor (AR), the Z gene *PKCIZ*, and W genes *ASW* and *CHDIW* (14, 15). The expression of Z and W mRNAs was compared in adjacent pairs of 10- μm coronal sections of the brain at various brain levels. AR mRNA expression was examined in several sections containing HVC. Sections were exposed to Kodak BioMax MR film, dipped in Kodak NTB-2 emulsion, and exposed for 7 days. A cDNA encoding the W gene *ASW* was isolated from a zebra finch hypothalamic library by using a chicken *ASW* cDNA generously provided by Andrew Sinclair (Royal Children's Hospital, University of Melbourne, Parkville, Victoria, Australia), then used to screen a 1-day-old male telencephalic library to isolate a cDNA encoding the Z gene *PKCIZ*, which shares significant homology to *ASW*. cDNA GenBank accession nos. are as follows: *PKCIZ*, AF539981; *ASW*, AF539982; and AR, AF532914. The *CHDIW* probe spanned nucleotides 5987–6324 of GenBank sequence AY217129. The AR probe used here was described in ref. 16.

Hormone Assay. Blood was drawn from the gynandromorph and from equivalently housed control males and females ($n = 8$ each). Plasma levels of steroids were measured by RIA (17).

Results

The gynandromorph had typical male plumage on the right side of its body with a characteristic orange cheek patch, thin black and white stripes on the neck, a solid black bar on the chest, and brown feathers with white spots under the wing (Fig. 1). The plumage on the left side lacked these characteristics and was typical of females. The gynandromorph's reproductive behavior was indistinguishable from that of a normal male. For example, when housed with a female, the gynandromorph often sang a fully masculine song (Fig. 2), courted, and copulated with the female. The female was fully stimulated by the gynandromorph and underwent several breeding cycles in which she copulated with the gynandromorph and laid and incubated several successive clutches of infertile eggs. When the gynandromorph was housed with a male, the male attacked it, suggesting that the gynandromorph was not attractive to the male.

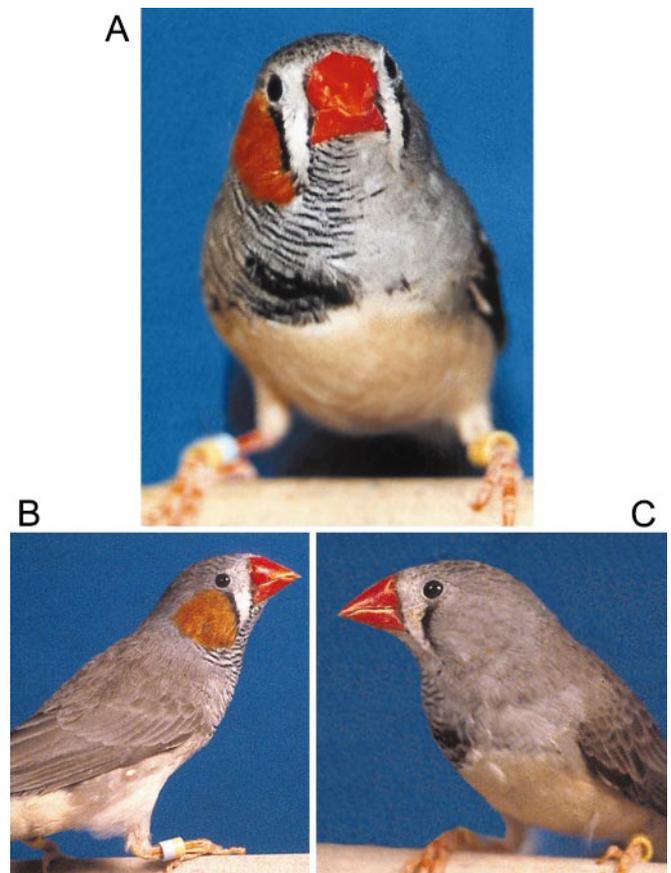


Fig. 1. Zebra finch gynandromorph (A) with male plumage on its right side (B) and female plumage on its left side (C). A few black male feathers can be seen on the left breast in A.

The phenotype and genotype of the bird were lateralized in numerous tissues. The gonadal phenotype was similar to bilateral gynandromorphs described (18–21). On the left side, the gonad was a histologically normal ovary with numerous normal and atretic follicles (Fig. 3 A and D); an oviduct was not apparent. On the right side was a mature but dysmorphic testis containing a mixture of large and small seminiferous tubules. The spermatogenic zone at the lining of some seminiferous tubules was disorganized and thin relative to that of normal males; nevertheless, sperm were observed in all stages of development (Fig. 3 A and B). Histological sections at the level of the gonads, but not more caudally, showed the presence of a deferent testicular duct on the right side (not shown). Plasma hormone levels of estradiol were below the detectable limit in all animals (less than ≈ 0.01 ng/ml). Testosterone was 0.11–1.26 ng/ml in seven control males but not detectable in the gynandromorph or control females (less than ≈ 0.2 ng/ml).

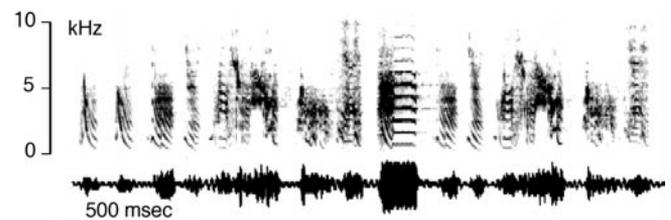


Fig. 2. Sound spectrogram and amplitude plot of the gynandromorph song show characteristics of normal male song: typical bout structure, with introductory notes and loud end notes, and typical frequency modulation.

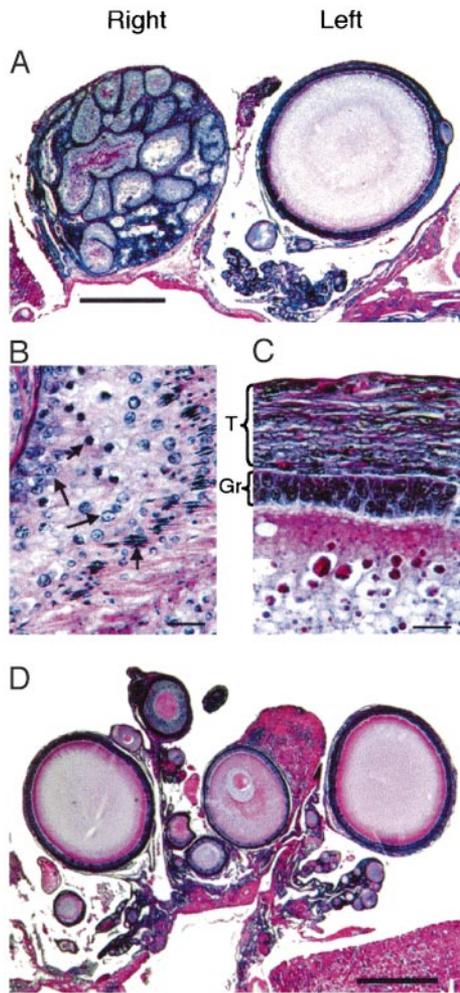


Fig. 3. Photomicrographs of histological sections of the gonads. On the animal's right side was a dysmorphic testis (A, bar = 0.5 mm) comprising a mixture of seminiferous tubules with unusually large or small lumens containing germ cells at all stages of spermatogenesis (arrows in B, bar = 20 μ m). Ovarian tissue was limited to the animal's left side and contained a number of follicles at different stages seen in two planes of section (A and D). A single mature follicle is seen in A, Left. Higher magnification (C, bar = 20 μ m) shows normal theca (T) and granulosa (Gr) cell layers.

The brain phenotype was examined by measuring sexually dimorphic regions in each hemisphere in Nissl-stained sections: neuron size and/or volume of the song nuclei HVC, RA, and Area X. For many of these measurements the two sides of the brain were similar (Table 1); however, nucleus HVC and, to a lesser extent, Area X had distinct lateral differences in volume. HVC was 82% larger on the right side (Fig. 4), a lateral difference 8.8 SD larger than the mean lateral difference in 17

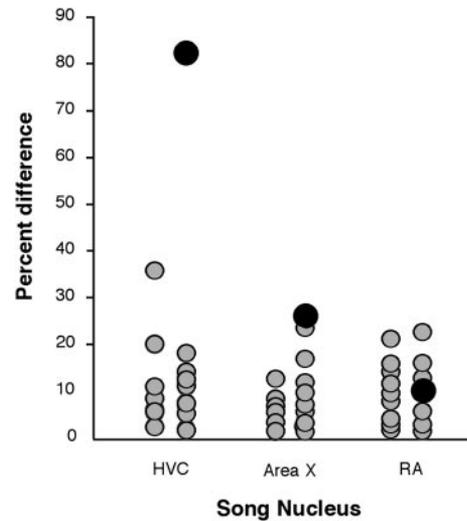


Fig. 4. The volume of song nucleus HVC was lateralized more in the gynandromorph than in normal males. Lateral differences in the gynandromorph song nuclei (black circles) were compared with lateral differences in multiple control males (shaded circles). For each nucleus, the left column represents males in which the left side was larger, and the right column represents values when the right side was larger. Percent difference is $(R - 1) \times 100$, where R = ratio of volume of larger side divided by volume of smaller side.

control males, which was $10.2 \pm 8.11\%$ (SD; range 1.0–35%; Table 1; refs. 9–11 and ||). The distribution of AR mRNA, a marker for HVC, confirmed the larger size of HVC on the right side (Fig. 5B). The absolute volumes of the right HVC, RA, and Area X were each on the low end of the range of volumes for control males, whereas the volume for nuclei on the left side was at or below the range for control males and well above the range for control females (Table 2). However, precise comparison of the absolute volumes should be avoided because of differences in tissue processing between the gynandromorph and control birds.

The expression of Z- and W-linked mRNAs showed a sharp division in expression of sex chromosome genes on the two sides of the brain. We measured expression of mRNAs encoding *CHDIW* (data not shown), *PKCIZ*, and *ASW* (14, 15) using *in situ* hybridization (Fig. 6). The sex chromosomes in male birds are ZZ and in females, ZW. The Z-linked *PKCIZ* mRNA, which is expressed in control male brains at higher levels than in female brains (data not shown), was widely expressed in the brain but higher on the right side (Fig. 6B). The W-linked mRNAs (*CHDIW* and *ASW*), expressed ubiquitously in control female brains (data not shown), were largely limited to the left half of the brain at different rostral to caudal levels, dramatically demonstrating a lateral difference in sex chromosome gene expression (Fig. 6A). This pattern is compatible with a ZZ genotype for the right side and ZW genotype for the left. A small

Table 1. Degree of laterality of the song system in gynandromorph and controls

	HVC			RA			Area X		
	Gyn	Male controls	Sample size	Gyn	Male controls	Sample size	Gyn	Male controls	Sample size
Volume ratio	1.82*	1.10 ± 0.08	17	1.10	1.07 ± 0.06	24	1.26*	1.05 ± 0.05	27
Volume range		1.01–1.35			1.00–1.22			1.00–1.23	
Neuronal soma size ratio	1.12	1.08 ± 0.08	27	1.02	1.07 ± 0.05	22			
Soma size range		1.00–1.25			1.00–1.19				

Ratios of volumes and neuronal cell sizes on the two sides of the gynandromorph (Gyn) and control males. Values are mean ratio of larger to smaller sides \pm SD. *Numbers were outside the range of controls.

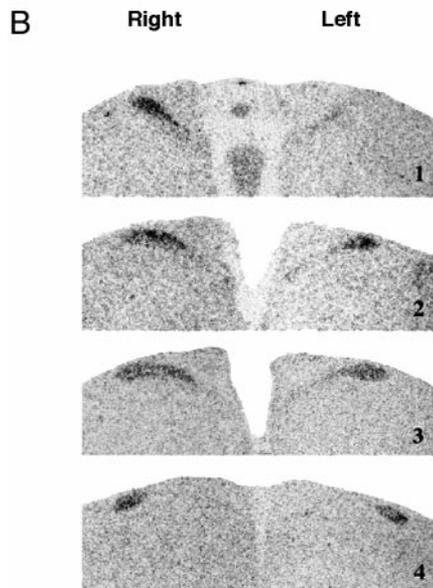
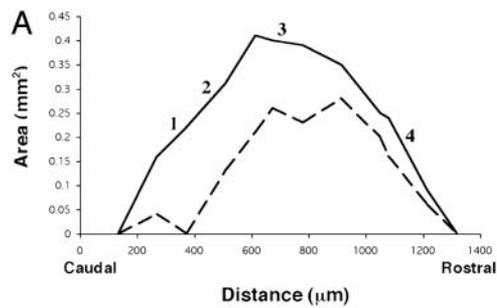


Fig. 5. (A) Graph of the area of HVC in individual Nissl-stained sections from the caudal to rostral extent of the nucleus, demonstrating a significant lateral difference in nucleus volume. Right is solid line; left is dashed line. (B) Inverted dark field autoradiograms of *in situ* hybridization showing the distribution of AR mRNA (dark areas) to mark HVC at various levels, confirming the lateral difference in HVC size. Numbers on the autoradiograms show level of sections on the graph.

number of *ASW*-expressing cells were found on the right side of the brain, especially near the midline (Fig. 7), suggesting that W-expressing cells had migrated a limited distance into the right side. Body tissues on both sides also appeared to differ by genotype based on the expression of W and Z genes. *In situ* hybridization of gonadal tissue with the *ASW* probe showed strong hybridization to follicles of the left ovary. In contrast,

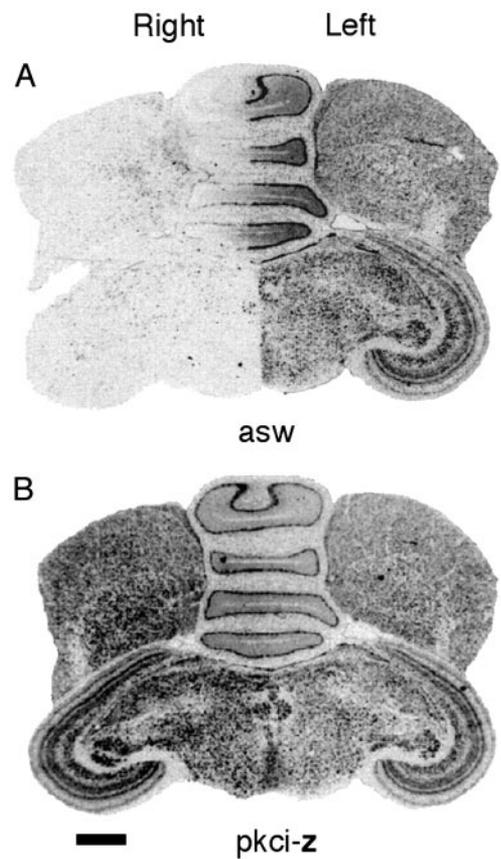


Fig. 6. Photomicrographs of *in situ* hybridization in brain sections using Z and W chromosome-specific probes. Autoradiograms were photographed in dark field and then black–white inverted; thus dark areas show label. (A) mRNA encoding the W chromosome gene, *ASW*, was ubiquitous on the left side but virtually absent on the right. The dividing line of high and low expression is sharp and follows the midline of the brain. (B) mRNA encoding the Z-linked gene *PKCIZ* was ubiquitous but higher on the right side of the brain, compatible with the idea that the brain was ZW on the left and ZZ on the right. (Bar = 1.0 mm.)

hybridization to testicular tissue on the right side was much reduced and was limited to the interstitial regions between the seminiferous tubules (data not shown).

Analysis of genomic DNA indicated that W genes were present at higher concentrations in the left half of the body. Using PCR to amplify distinguishable genomic fragments of the related Z and W chromosome genes *CHDIZ* and *CHDIW*, we

Table 2. Absolute volumes of song nuclei in gynandromorph and controls

	HVC volume	Sample size	RA volume	Sample size	Area X volume	Sample size
Gyn						
Right side	0.295		0.171		1.18	
Left side	0.162		0.156		0.939	
Male						
Controls	0.414 ± 0.11	17	0.288 ± 0.06	24	1.46 ± 0.24	27
Range	0.268–0.703		0.160–0.386		1.10–1.93	
Female						
Controls	0.057 ± 0.02	26	0.039 ± 0.01	28	0	
Range	0.020–0.106		0.020–0.106		0	

Absolute volumes of individual song nuclei of the gynandromorph (Gyn) and control birds. Values are given as the mean (mm³) ± SD. The right brain nuclei of the gynandromorph were at the lower end of the range of control males but well above the range for females. The left brain nuclei were at or below the lower end of the range for control males but well above the range for females. Gynandromorph and control tissues were processed differently; thus precise comparison of absolute volumes is not warranted.

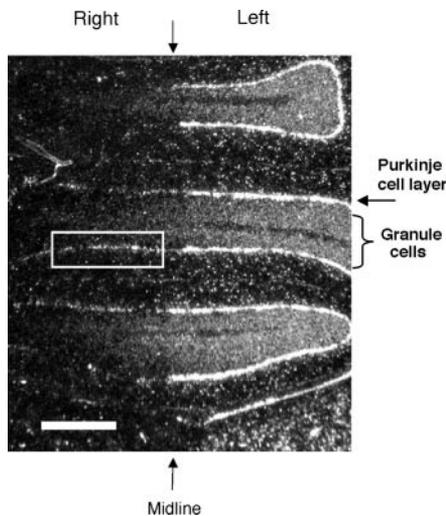


Fig. 7. Dark-field photomicrograph of autoradiogram showing *in situ* hybridization of a probe recognizing *ASW* in the cerebellum. The uniformly high expression of *ASW* mRNA can be seen in left-side Purkinje cells and granule cells, extending to the midline (arrows). Labeling for *ASW* decreased abruptly on the right side beginning at the midline. However, some Purkinje cells were labeled for *ASW* on the right side (box), and granule cells closer to the midline were labeled more than those farther from the midline. (Bar = 0.5 mm.)

found that *CHD1Z* was amplified from genomic DNA extracted from both sides of the brain or feather pulp, but *CHD1W* was prevalent only on the left side (Fig. 8A). In Southern blot analysis, the *W* gene *ASW* was detected more strongly in DNA from left muscle and feather pulp than from right (Fig. 8B). Because the DNA content of blood cells was intermediate to that of normal males (ZZ) and females (ZW), the gynandromorph was likely not polyploid or ZZW aneuploid (Fig. 9).

The syrinx, which is also sexually dimorphic, was found to be intermediate between a normal male's and female's in overall thickness of the syringeal muscles (Fig. 10), suggesting that the forces that cause sexual differentiation of this tissue were also intermediate. We did not characterize any lateral differences in genotype of the syrinx, and the large variability in muscle fiber size on each side made conclusions regarding right-left differences difficult.

Discussion

The avian gonad sexually differentiates according to a cell-autonomous genetic program determined by a gene or genes on

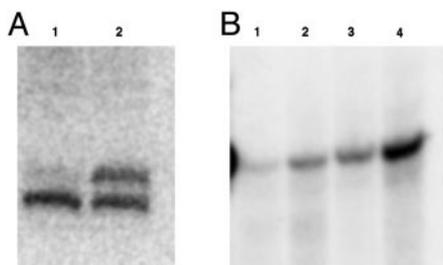


Fig. 8. Analysis of lateralization of sex chromosomes. (A) PCR products representing fragments of *CHD1Z* and *CHD1W* amplified from genomic DNA from the right (1) and left (2) sides of the gynandromorph brain. *CHD1W* (upper band) was predominantly restricted to the left side of the brain, suggesting a lateralization of the *W* sex chromosome. *CHD1Z* (lower band) was found on both sides of the brain. (B) Southern blot of genomic DNA from leg muscle (1, right, and 2, left) and feather pulp (3, right, and 4, left) of the gynandromorph shows greater amount of *ASW* in genomic DNA from the left side than from the right.

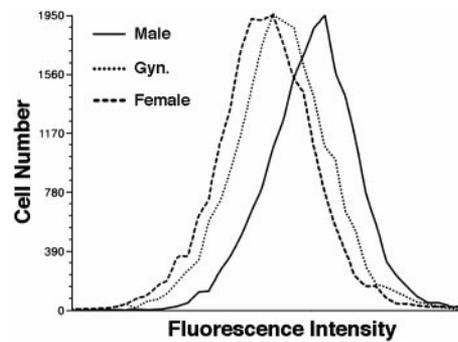


Fig. 9. Measurement of DNA content of blood cells. Graphs compare the distribution of intensity of propidium iodide labeling of nuclear DNA in populations of blood cells from the gynandromorph and a typical control male and female. Blood DNA content of the gynandromorph was intermediate to control males and females, which suggested the animal was within the diploid range. The male and female plots represent five individuals for each.

the *Z* or *W* chromosomes. Similarly, sexual differentiation of zebra finch plumage is under cell-autonomous genetic control because the sexual phenotype of plumage always correlates with the genetic sex of the gonads, even in numerous experiments in which zebra finch eggs or young have been treated with various gonadal hormones or inhibitors of hormones (22). In the gynandromorph, the female sexual phenotype of the left plumage and gonad correlated well with the left-side predominance of two *W*-linked genes (*CHD1W* and *ASW*) as determined by genomic PCR, Southern blot, and expression of *W*-linked genes shown by *in situ* hybridization. Thus the genetic mechanisms controlling sexual differentiation in the gynandromorph seem to differ on the two sides of the body at various levels, with the left half containing the genes leading to female differentiation and the right half containing genes leading to male differentiation.

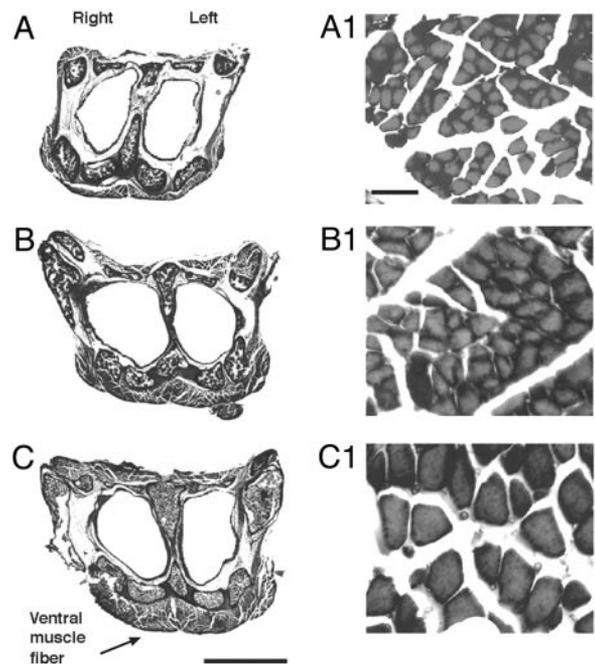


Fig. 10. Photomicrographs of transverse sections through the syrinx at the level of the pessulus (A–C, bar = 1.0 mm) and higher magnification of ventral muscle fibers (A1–C1, bar = 20 μ m). Female control (A), gynandromorph (B), and male control (C). Overall, the thickness of the syringeal muscles and of individual fibers in the gynandromorph was intermediate to that of males and females. The genotype was not determined for the two sides of the syrinx.

A lateral division of sexual genotype in the brain was suggested by genotypic and phenotypic characteristics. Genomic PCR showed a left-side predominance of the W gene *CHDIW*, and *in situ* hybridization indicated that expression of W-linked *ASW* mRNA was also predominantly in cells on the left side. Moreover, the Z-linked mRNA *PKCIZ* was expressed at a higher level on the right side, as would be expected from a ZZ sex chromosome genotype. The expression data therefore support the interpretation that W- and Z-linked genes were represented differentially in the genome on the two sides of the brain.

The sexual phenotype of the gynandromorph's brain also was lateralized in the sexually dimorphic song regions HVC and Area X. HVC was much larger on the genetically male side of the brain compared with the female side. This difference was far outside the range of the lateral differences found in the HVC of control males. The lateral difference in Area X volume was also outside the normal range, but less so. As with plumage and gonadal differences, these lateral differences in the brain are attributable to a lateralization of cell-autonomous genetic factors, probably those encoded on the sex chromosomes. Because the two sides of the brain would have been equally exposed to circulating gonadal hormones throughout development, the unequal sexual differentiation of the two sides of the brain cannot be attributed solely to exposure to gonadal hormones. Rather the differences reflect endogenous genetic differences in the brain cells themselves.

Bilateral gynandromorphism has been reported previously in birds, but the developmental error responsible is unknown (18–21). It could have resulted from failure of polar body extrusion during egg meiosis, producing a binucleate ovum with Z and W nuclei that were each fertilized by separate sperm (23). The error seems to have been present at the two-cell stage and preserved thereafter during separate development of the two halves of the bird. The division of genetic sex was not perfect. Some W chromosome DNA was found in the right brain, muscle, and feather pulp (Fig. 8). Small numbers of cells expressing W genes were found on the right side of the brain, especially close to the midline (Fig. 7). Similarly, a few feathers with male phenotype were found on the left breast (Fig. 1). This pattern suggests that cells originating on each side migrated across the midline in small numbers at later stages of development. This observation and the incomplete removal of blood from the right side during perfusion likely accounts for the detection of some W chromosome DNA on the right side of the bird.

Soma size in all three song regions and the volume of RA did not differ on the two sides of the brain. This finding suggests that some parts of the masculine phenotype of the song circuit may be more

sensitive to diffusible factors than others. The estrogen dependence of masculine ingrowth of axons into RA (24) may have made differentiation of RA especially sensitive to hormonal factors.

Although HVC and Area X were more masculine on the right side, the song nuclei were bilaterally more masculine than the nuclei of control females. Possible explanations are that the right brain masculinized the left via transsynaptic trophic interactions (25, 26), or that the small testis secreted hormones that masculinized both sides. However, we favor a mechanism whereby diffusible factors such as steroid hormones, originating in the brain itself, masculinized both sides (27). This mechanism agrees with the finding that estrogen-treated females have a significantly masculinized song system (6), and that estrogen synthesized in male brain tissue can masculinize parts of the female song system *in vitro* (24). Interestingly, masculinization of the gynandromorph's song system occurred in the presence of both testicular and ovarian tissue, yet in genetic females also containing both gonadal tissues, or almost exclusively testicular tissue, the song system is not masculinized (7). These findings suggest that the presence of genetically male brain tissue is required for the significant masculinization seen on both sides of the brain, perhaps because of neural synthesis of estrogen or other hormones. In that case cell-autonomous gene actions would be required to explain lateral differences in hormone production in the brain of this bird.

These results provide the strongest evidence to date that sex differences in the song circuit of zebra finches originate partly because of differences in the actions of sex chromosome genes acting locally within the brain. This system thus represents one of a growing number of model systems in which the actions of sex chromosome genes are implicated in sexual differentiation of nongonadal tissues in birds and mammals. Other sexually dimorphic phenotypes influenced by the genetic sex of cells include the size of mammalian embryos (28), external genitalia of tammar wallabies (29), aggression in mice (30, 31), phenotype of midbrain and hypothalamic cells in rats and mice (32, 33), and vasopressinergic innervation of the lateral septum of mice (34). Further work is needed to resolve how the hormonal and cell-autonomous mechanisms interact to produce sex differences in brain phenotype and disease.

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