

Generating sexually differentiated songs

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The past year has witnessed increased confusion as to the role of gonadal hormones in the development of neuroeffectors for sexually differentiated vocalizations in several species. Are sex differences in levels of circulating gonadal hormones robust enough to account for the full spectrum of male/female differences? Understanding how vocal behaviors are generated has improved, permitting greater insights into how differences in cell number and type contribute to male- and female-specific songs in frogs and birds.

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Abbreviations

EOD	electric organ discharge
HVc	higher vocal center
LM	laryngeal-specific myosin heavy chain
RA	robustus archistriatalis

Introduction

The focus of this review is on how sexually differentiated motor programs are generated. Courtship vocalizations, in particular the songs of birds and frogs, have provided experimental fodder for this question. Two current issues are the relation between hormones and masculinization of vocal systems and the relation of cell number and type to the production of specific vocal features.

Sexual differentiation

Adults generate sexually differentiated motor programs that underlie male- and female-specific courtship, reproductive and parental behaviors [1]. Two distinct developmental mechanisms, 'genetic' and 'hormonal', are commonly thought to account for sex differences. For example, in the fruit fly (*Drosophila melanogaster*), all cells 'read' the sex chromosome-to-autosome ratio and adjust their patterns of gene expression accordingly using sex-specific splicing of messenger RNA to generate distinct male or female biochemical environments in every cell [2]. In mice (and probably humans as well), chromosomal mechanisms (i.e. the *sry* gene on the Y chromosome) control only the developing gonad [3]. Once gonadal sex is determined, the 'message' of femaleness or maleness is conveyed by hormones secreted by ovaries or testes and received by distant cells that express matching intracellular receptors [4].

How do neuroeffectors for sex-specific behaviors differentiate?

The CNS is a major target for sex hormones, as are certain specialized muscles devoted to reproductive behaviors [1]. Many of these hormone targets differ dramatically—in number, size and type—between males and females, and they are strongly affected by manipulating the endocrine state of young or adult animals. Parallels between morphological, biochemical and behavioral changes induced by hormone manipulations in rodents and primates gave rise to the prevailing concept that male- and female-specific behaviors can be traced to sex differences in hormones secreted by the gonads—androgens, estrogens and progestins—during development and in adulthood (the 'mammalian model'). This orthodox viewpoint is now being challenged by studies in other vertebrates, suggesting that gonadal secretion may not be sufficient—or even necessary—for secondary sexual differentiation and raising the possibility that more direct genetic mechanisms operate in vertebrates as well as in invertebrates.

At first glance, masculinization of the song system in the South African clawed frog (*Xenopus laevis*) appears to follow the mammalian model: exogenous androgens masculinize a motor control system that includes the vocal organ or larynx and laryngeal motor neurons [5]. As antiandrogens in males block, and testicular transplants in females induce, masculinization of motor neurons and muscle fiber numbers, endogenous androgens appear necessary for masculinization. However, endogenous androgen levels in males and females are not very different during development, and androgen of adrenal origin is present even before the gonads form [6]. Sex differences in circulating androgen levels may not be sufficient to account entirely for masculinization. Also, exogenous androgens do not mimic the effects of testicular transplants on cell numbers [7,8], though they do masculinize cell type [9]. Androgen-treated females do not produce the male vocal pattern, whereas testis-transplanted females do. Androgen-treated females have bigger vocal motor neurons and muscle fibers and have all fast twitch muscle fibers, but their motor neuron number and laryngeal muscle fiber number are still female-like. Both cell number and type are masculinized in testis-transplanted females; thus, cell number is more closely correlated with the ability to produce male vocal behaviors than is cell type.

The testis may be more than an androgen pump. For example it may secrete hormones other than steroids that are essential for masculinization or express a pattern of hormone secretion not mimicked by steroid implants. Marked sex differences in the expression of androgen receptors in the vocal system are also present and

parallel the state of proliferation and differentiation of laryngeal cells [10–12,13*]; sex differences in sensitivity to, rather than secretion of, androgen may be key for sexual differentiation. What in turn controls sexually differentiated receptor expression is a mystery. In frogs, even though males are the homogametic sex (ZZ) [5], a simple genetic explanation (e.g. androgen receptor on the Z chromosome) does not fit with the observation that levels of androgen receptor expression are the same in males and females before masculinization begins [10,11]. Steroid receptor expression, however, has multiple levels of control that include transcription, splicing, mRNA and protein stability, nuclear translocation, dimerization and association with co-factors [4]. These regulatory steps are candidate mechanisms for sexually differentiated sensitivity to steroid hormones.

The search for the hormonal basis of sexual differentiation of the song system in bird forebrain has provided an even more radical challenge to the mammalian model [14]. The song system consists of a series of interconnected forebrain nuclei (cytoarchitecturally distinct brain regions) that access the vocal motor neurons projecting to the syrinx (vocal organ) via projection neurons in nucleus RA (robustus archistriatalis). In many oscine (song) birds, all forebrain song nuclei are larger in volume in adult males [15] because they have more cells and/or increased neuropil [16–18]. The song nuclei of female zebra finches can be masculinized by applying exogenous estrogen around the time of hatching [16,18,19]; these females go on to produce male-like songs when they become adults [18–20].

Estrogen is a testosterone metabolite; intracellular conversion (aromatization) of testicular derived testosterone to estradiol acts to masculinize brain nuclei associated with copulatory behavior in rodents via binding to the estrogen receptor [14]. The demonstration that the bird forebrain has very high levels of aromatase activity, sufficient to influence circulating estrogen levels elsewhere in the body, supported the idea that masculinization in birds followed the mammalian model [21]. However, estrogen has another established role in bird sexual differentiation: it demasculinizes male copulatory (mounting) behaviors [20]. Both effects are observed during the same developmental period [22]. To date, no treatment designed to decrease levels of endogenous estrogen (including antiestrogens and castration) has been effective in demasculinizing the song system of males [23–25]. At stages in which exogenous estrogen masculinizes the song system, there are no sex differences in the levels of circulating androgens (the substrate for aromatase), circulating estrogens (mostly derived from brain aromatization), or levels of aromatase itself. The song nuclei themselves do not express the aromatase gene [25]; of the song nuclei, only the forebrain higher vocal center (HVC) expresses significant amounts of the estrogen receptor, and the levels are the same in both sexes [14,26].

Recent studies have cast additional doubt on whether testicular tissue contributes at all to sexual differentiation of the song system. If developing zebra finches are treated *in ovo* with the aromatase inhibitor fadrozole, genetic females develop testicular tissue that secretes androgens, but the song nuclei are not masculinized [27**,28]. In genetic males, the song nuclei remained masculinized after treatment despite a severe decrease in the levels of brain estrogen. Taken together, the results suggest that steroid hormones from the gonad or from the brain do not govern masculinization of the bird song system.

So what does account for brain masculinization in songbirds? One possibility is that the presence of any ovarian tissue blocks the masculinizing action of the testes: exogenous estrogen could act by interfering with this non-estrogen ovarian factor [23*,27**,28]. A more extreme hypothesis [29] is that masculinization and feminization do not result from sex differences in circulating hormones but from a more direct genetic mechanism: for example, expression of certain genes on sex chromosomes in the CNS. At present, although there is a great deal of evidence that steroid hormones play some role in the sexual differentiation of the vocal system in songbirds, the native mechanisms involved have not yet been characterized.

Not all mammals conform to the mammalian model. In the tammar wallaby, some aspects of the development of secondary sexual traits do not depend on gonadal steroids; instead, they are associated with sex chromosomes: the scrotal and mammary anlage differentiate before the gonads form and are thus not regulated at this early stage by sex steroids [30]. It is not yet clear whether these different patterns of sexual differentiation reflect highly species-specific developmental mechanisms or whether mechanisms exaggerated in other vertebrates and eutherian mammalian systems are also present in lab mammals; we may have to reexamine the mammalian hypothesis to see if sex differences in levels of circulating gonadal hormones are truly robust enough to account for the full spectrum of male/female differences.

Generating male-specific motor programs

One of the reasons why attention is paid to cell number in song nuclei is its strong correlation with ability to produce masculine behaviors. In both birds and frogs, females with masculinized cell numbers produce male songs, whereas those with only masculinized cell size or type do not [19,31]. How having more cells actually contributes to behavioral expression is not clear, mostly because how the motor programs are executed is not known in detail. The past two years have seen significant improvement in our understanding of how songs are generated by the CNS and muscles, and how hormones affect activity, down to the level of channel kinetics.

In the songbird CNS, the hierarchical roles of RA and HVC in song production have been clarified. Syringeal

muscles are topographically represented in the motor nucleus [32] and, in turn, in RA [33]. Electrical stimulation of HVc distorts syllables and song patterning, whereas RA stimulation has only the first effects [34]. A major advance has been recordings from HVc and RA of singing birds [35**], showing that HVc activity is tied to syllabic organization, whereas RA activity is tied to the note, a sub-syllabic acoustic event. Chronic recordings from the syrinx of singing birds have demonstrated that airflow through each side of the syrinx can be independently controlled and that dorsal and ventral muscles differ in patterned activity during song [36]. Song production by the syrinx is lateralized [37]. Recordings from singing brown thrashers (whose size permits simultaneous recording from syringeal muscles and monitoring of airflow in the bronchi) reveal that only the dorsal muscles participate in gating airflow [38]. The ventral muscles, which are more numerous, are active on both sides, an observation that may help resolve a long-standing puzzle of why the bird CNS song system is functionally but not morphologically lateralized. The control of phonation is complex and the activity of the four pairs of ventral muscles can now be tied to specific synergistic and antagonistic actions, sounds produced and phases of respiration [39*].

Recordings from forebrain structures and syrinx have been carried out only in male birds. We do not yet know whether the vocal organ itself (the syrinx in birds can be highly dimorphic in size within the sexes) contributes to sex differences in vocal behaviors, as is the case in other song systems. In *Xenopus laevis*, male and female vocalizations differ markedly in temporal pattern, rate and duration [40]. Nerve stimulation of the isolated larynx produces sounds that closely resemble actual calls [41]. When the male larynx is stimulated via the nerve with trains whose temporal features mimic the mating call, the male larynx produces this advertisement call; when the female larynx is stimulated with trains that mimic ticking, the female larynx produces this release call. The female larynx cannot produce mating calls. Thus, this *vox in vitro* preparation provides a physiological link between sex-specific songs and hormonally driven cellular and molecular mechanisms.

While the temporal pattern is generated by the CNS, cellular and molecular characteristics of laryngeal muscle fibers and the laryngeal synapse impose sex-specific limitations on sound production [41]. Female muscle fibers cannot generate the rapid trills of male mate calling because they are mostly slow twitch [8]. Males can generate rapid contractions (up to 100 Hz); this ability is associated with expression of a laryngeal-specific myosin heavy chain gene (LM) [42]. Recent studies suggest that the developmental transition in males from slow to fast twitch fibers [43] relies on androgen regulation of LM expression [44]. Unlike females, males cannot generate muscle action potentials in response to single shocks delivered to the laryngeal nerve; instead, they

rely on facilitation to trains of activity to produce muscle contractions and trills [45].

Quantal analyses of vocal synapses have localized the sex difference in synaptic efficacy to the presynaptic terminal of the motor nerve; males release less neurotransmitter than females [46]. The subthreshold, facilitating laryngeal synapses of males are associated with the production of amplitude-modulated trills, another sexually differentiated trait [45]. Through juvenile stages, both male and female vocal synapses are subthreshold (i.e. quantal content is low or male-like); synaptic strength increases at reproductive maturity in females, probably in response to estrogen secretion [47]. As laryngeal motor neurons do not appear to express estrogen receptor [48], the strengthening of the synapse could be attributable to a retrograde signal from the muscle fiber. The molecular mechanisms for weak-male and strong-female vocal synapses are unknown; candidate mechanisms must take into account the recent observation that facilitated release evoked by paired pulses or trains of stimuli is the same in both sexes [49*].

Further cellular insights into hormone action on motor systems have come from the sexually differentiated 'songs' of weakly electric fish; electric organ discharges (EODs) produced by electrocytes, modified muscle cells [50]. In *Sternopygus*, male EODs are low frequency, whereas female EODs are high frequency; androgen lowers EOD frequency in females and increases the action potential duration of electrocytes [51]. EOD frequency reflects kinetic features of the sodium current: androgen treatment raises inactivation time constants in electrocytes [51], whereas estrogen treatment lowers them [52*]. A decrease in the inactivation time constant of the sodium current should raise firing rates, whereas an increase should depress firing. This has been observed even when electromotor neurons and their afferents were isolated in an *in vitro* slice preparation [53**]. Independent, opposing actions of androgens and estrogens on motor neurons and muscle cells may underlie sexually dimorphic features of the electric communicatory signal.

Future directions – females that sing

Understanding how sexually differentiated behaviors are generated has profited from models of courtship song in which sex differences are dramatic: males sing and females do not. Paradoxically, this dramatic difference has proved a limitation. Identifying which of the very large number of sex differences present in song systems are key to male-specific motor programs has been difficult because females typically have all or most of the effectors—neural and muscular—used to produce song, albeit in different or diminished form. A system in which the sexes sang songs that differed in a number of ways (not simply that one sex sings while the other does not) would permit a more refined understanding of the relation between cellular and behavioral sex differences.

Newly discovered songs in female *Xenopus* could provide an opportunity to tackle the difficult question of how sex differences at the cellular level contribute to different motor programs at the behavioral level. When female *Xenopus* are about to oviposit, they produce a fertility advertisement call, rapping, that evokes intense calling and attempted copulation in males [54**]. The non-ovulating, sexually unreceptive female produces a different call, ticking [40], but can be switched from ticking to rapping within 6 hours following gonadotropin injection [54**]. The discovery of rapping provides a rich arena in which to examine the cellular control of vocal behaviors, such as whether the properties of the laryngeal synapse are altered during the switch between ticking and rapping.

An intriguing aspect of vocalizations in birds is sex differences in learning. In the zebra finch, both sexes produce the long call that contains learned components in males but not females. Production of the long call itself does not rely on the HVC-RA-syringeal-motor-neuron pathway; acoustic features that reflect learning do require this pathway [55]. Early estrogen treatment causes females to produce male-like learned call features that are abolished by lesion of this pathway [56]. Which aspects of brain sex differences are responsible for the alteration of vocal behaviors by auditory experience in one sex but not the other are not known. Duets sung by male/female pairs are present in over 100 species of songbird [57]; male/female duets offer another potential system in which to examine these questions. Males and females usually sing sex-specific songs, but can learn the song of the other sex if reared in isolation [58]. Given recent progress in recording from song nuclei of behaving birds, the stage is set for further study of sex differences in learning both of long calls and of songs produced during duets.

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