Neuroeffectors for Vocalization in *Xenopus laevis:* Hormonal Regulation of Sexual Dimorphism

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Received January 23, 1986

SUMMARY

South African clawed frogs use sex-specific vocalizations during courtship. In the male, vocalizations are under the control of gonadal androgen. Though females have moderate levels of circulating androgen, they do not give male-typical mate calls. Both muscles of the vocal organ and neurons of the central nervous system (CNS) vocal pathway are sexually dimorphic and androgen-sensitive. Recent studies suggest that the failure of androgen to masculinize adult females results from a male-specific, androgen-regulated developmental program. At metamorphosis the larynx is sexually monomorphic and feminine in morphology, muscle fiber number and androgen receptor content. During the next six months, under the influence of increasing androgen titers and high receptor levels, myoblasts proliferate in the male and muscle fibers increase at an average rate of 100/day. Females have much lower hormone levels, receptor values decline and they display no net addition of fibers. At metamorphosis, both males and females have approximately 4000 muscle fibers. By adulthood, males have eight times the female fiber number. In the CNS, adult laryngeal motor neurons are more numerous with larger somata and dendritic trees in males than in females. Certain connections of neurons in the vocal pathway are also less robust in females. Unlike the periphery, motor neuron number does not appear to be established by androgen-induced proliferation. Our current hypothesis is that androgen acts at the level of laryngeal muscle to produce more muscle fibers and thus provide more target for motor neurons in the male. This process could regulate cell number by ontogenetic cell death. In the CNS, androgen-target neurons become capable of accumulating hormone shortly before metamorphosis. Androgen receptor in larygeal motor neurons may permit the dendritic growth characteristic of males by increasing sensitivity to afferent stimuli. Such a process could account for the observed differences in CNS vocal "circuitry" in *X. laevis* and thus behavioral differences between the sexes.

INTRODUCTION

The vertebrate brain is sexually dimorphic. Sex differences result from sex-specific patterns of secretion of gonadal steroids. The action of sex hormones on neuronal differentiation is considered to operate via two distinct, age-dependent processes. During perinatal development, steroids cause ir-

Journal of Neurobiology, Vol. 17, No. 3, pp. 231–248 (1986)
 CCC 0022-3034/86/030231-18$04.00

reversible changes in the number, morphology and function of certain groups of neurons. After sexual maturity, sex hormones develop or amplify the sexual differentiation of target neurons. These processes have been called the "organizational" and the "activational" effects of hormones, respectively (Phoenix et al., 1959; Feder, 1981). Organizational effects are permanent and operate during certain limited "critical" or sensitive periods. Activational effects are reversible and operative throughout adult life.

A necessary condition for the action of steroids on target cells is the presence of intracellular receptor proteins. Mutants lacking or defective in receptors do not exhibit full sex-typical differentiation (Wilson et al., 1981). Neurons exhibiting male or female specific functional or morphological differentiation have large amounts of appropriate hormone receptors. It has seemed reasonable to suppose that sex hormones act directly, via steroid receptors, on their target neurons to cause sexual differentiation. One well established aspect of neuronal differentiation, however, is the dependence of neurons on afferent input and on retrograde influences from targets. A relevant example is ontogenetic cell death, a process during which more neurons are born than will survive to maturity; in many systems approximately 50% of these cells die during the developmental period of synapse elimination (Hamburger, 1975). Cell death is regulated by interactions with the periphery including target size (Hamburger, 1975) and activity (Pittman and Oppenheim, 1979). Afferents also contribute to cell survival during the critical period of ontogenetic cell death (Oppenheim, 1984). It is thus reasonable to ask whether the entire process of neuronal sexual differentiation is due to direct action on target cells of whether the process is instead (or in addition) run by steroid action on afferents or efferent targets.

Recent research from our laboratory and results from other dimorphic, neuromuscular systems suggest that central nervous sexual differentiation depends on hormone action at the periphery. For example, male rats (and men) possess a set of muscles (the levator anti-bulbocavermosus complex) and associated SNB motor neurons that females lack (Cihak et al., 1970; Forger and Breedlove, 1985). The muscles are present in both sexes at birth but involute in females unless exogenous androgen is supplied (Venable, 1966; Breedlove and Arnold, 1983). SNB motor neurons are also present in both sexes before birth but die during post-natal development without androgen (Sengelaub et al., 1985). Both muscle and motor neurons contain large amounts of androgen receptor (Bleisch et al., 1982; Arnold and Saltiel, 1979). Androgen rescues female SNB neurons even when these are cut off from input from anterior or posterior regions of the neuraxis (Fishman and Breedlove, 1985). It is possible to localize the effective site of androgen rescue (motor neuron versus muscle) by use of mutant mice. Breedlove crossed *Sxr* to *Tfm* mice and observed that rescued SNB motor neurons did not contain androgen receptor (Breedlove, 1986). One reasonable explanation of these elegant results is that androgen acts on muscle to save motor neurons and does not act directly on the motor neurons. It remains to be determined whether other sexually differentiated characteristics of the motor neurons (e.g., dendritic length, synaptic connectivity) require androgen receptor activation for permissive or inductive effects on sexual differentiation.

The goal of our studies is to determine how androgenic steroids control the differentiation of target muscles and how the resultant masculinized periphery contributes to the development of sex differences in the central

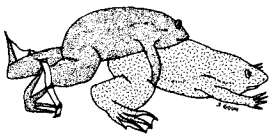
nervous system. The focus of the work is on development of muscle and the neuromuscular junction in the larynx of *Xenopus laevis,* the African clawed frog. Several features of this system make it particularly favorable for studies of sexual differentiation. Sex differences are marked and present in both periphery and central nervous system. Unlike the *levator ani,* the female larynx perists into adulthood and is functional; "organizational" and "activational" effects of androgen can thus be readily distinguished. All stages of development are readily accessible to hormonal manipulation (this is not the case *in ovo* or *in utero*). Yet, since the program for sexual differentiation in *Xenopus laevis* has many features in common with other systems, mechanisms uncovered may be widely applicable.

The present review describes current knowledge of the neural and muscular mechanisms for vocalization in *Xenopus laevis,* the multiple sexual dimorphisms of this system, its endocrine regulation and development. My aim is to place our particular results in the general context of cellular mechanisms which govern the differentiation of nervous systems.

VOCAL BEHAVIORS

African clawed frogs, *Xenopus laevis,* use at least three distinctive calls in social communication: mate calls, ticking, and sawing. Males mate call to attract and excite females (Russell, 1954; Wetzel and Kelley, 1983). The mate call is a repetitive trill, composed of brief clicks (Fig. 1). Each mate call is made up of a fast and slow trill portion chained together in repetitive bouts. The mean interclick interval (ICI) is 14 msec for the fast and 28 ms for the slow portions of the trill (Wetzel and Kelley, 1983), an extremely rapid rate of vocal production.

Ticking, first described by Russell (1954), is a call given by sexually unreceptive females when clasped or in response to the approach of a vocalizing male (Kelley, 1982; Kelley and Long, unpublished). Ticking is also given



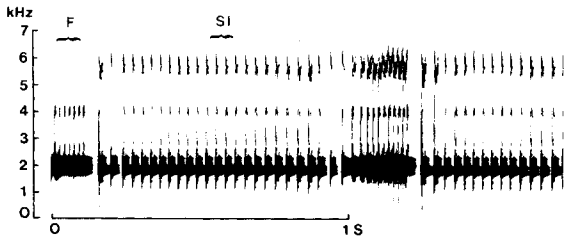


Fig. 1. Mate calling, a male characteristic vocal pattern in *X. laevis*. The upper panel shows a sexually receptive female frog being clasped by a male (from Kelley, 1982). A sonogram of a male mate call is shown below. The call consists of alternating fast (F) and slow (Sl) trill phases. The interclick interval in the slow trill phase is 28 ms and in the fast trill phase is 14 ms (data from Wetzel and Kelley, 1983).

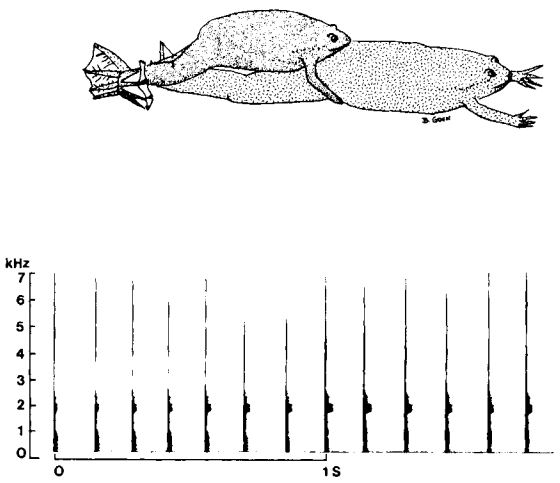


Fig. 2. Ticking, a vocalizaton given by sexually unreceptive female *Xenopus laevis*. The upper panel shows an unreceptive female being clasped by a gonadotropin injected male (from Kelley, 1982). A sonogram of ticking is shown below. The call is a very slow trill and consists of regularly repeated clicks with an interclick interval of approximately 165 ms.

by males when clasped by other males. The vocalization, like mate calling, is made up of trains of clicks (Fig. 2). The temporal patterning of the clicks is, however, distinctive in that the trill rate is slow (ICI 165 ms) and regular (i.e., no fast and slow trill portions). The clicks comprising mate calls in males and ticking in females are acoustically distinguishable; those of males contain a higher proportion of high frequency components (>1.7 kHz; Hannigan and Kelley, 1986). Ticking by the female in conjunction with leg extension and movement induces the clasping male to release, usually within a few seconds (Kelley, 1982; Weintraub et al., 1985). The third vocalization, termed "sawing" by Russell (1954), is given when sexually active males or androgen-treated females are paired (Kelley and Long, unpublished) and is believed to function as an aggressive signal (Rabb, 1973).

MECHANISMS OF VOCAL PRODUCTION

Vocalizations are produced within the larynx (Ridewood, 1898; Yaeger, 1982; Tobias and Kelley, 1985). This box-like structure of cartilage and muscle is located immediately dorsal to the heart. The larynx gains access to the buccal cavity via the glottis, located anterior and dorsal to the vocal organ, and to the lungs via the proximal portions of the two tracheae, located at the posterior pole (Fig. 3). The larynx is made up largely of hyaline cartilage and muscle; the cartilage forming an elaborate chamber enveloped by two bilaterally symmetric muscle sheets, the *m. dilator laryngis* (Ridgewood, 1898; Yaeger, 1982). At the anterior end of the larynx are located paired arytenoid discs resembling bell clappers (Fig. 3C). The discs are connected, via an anteriorly-posteriorly running tendon, to the laryngeal dilator muscle and when the latter contracts the discs are pulled apart. The resultant movement is responsible for click production (Ridewood, 1898; Yaeger, 1982).

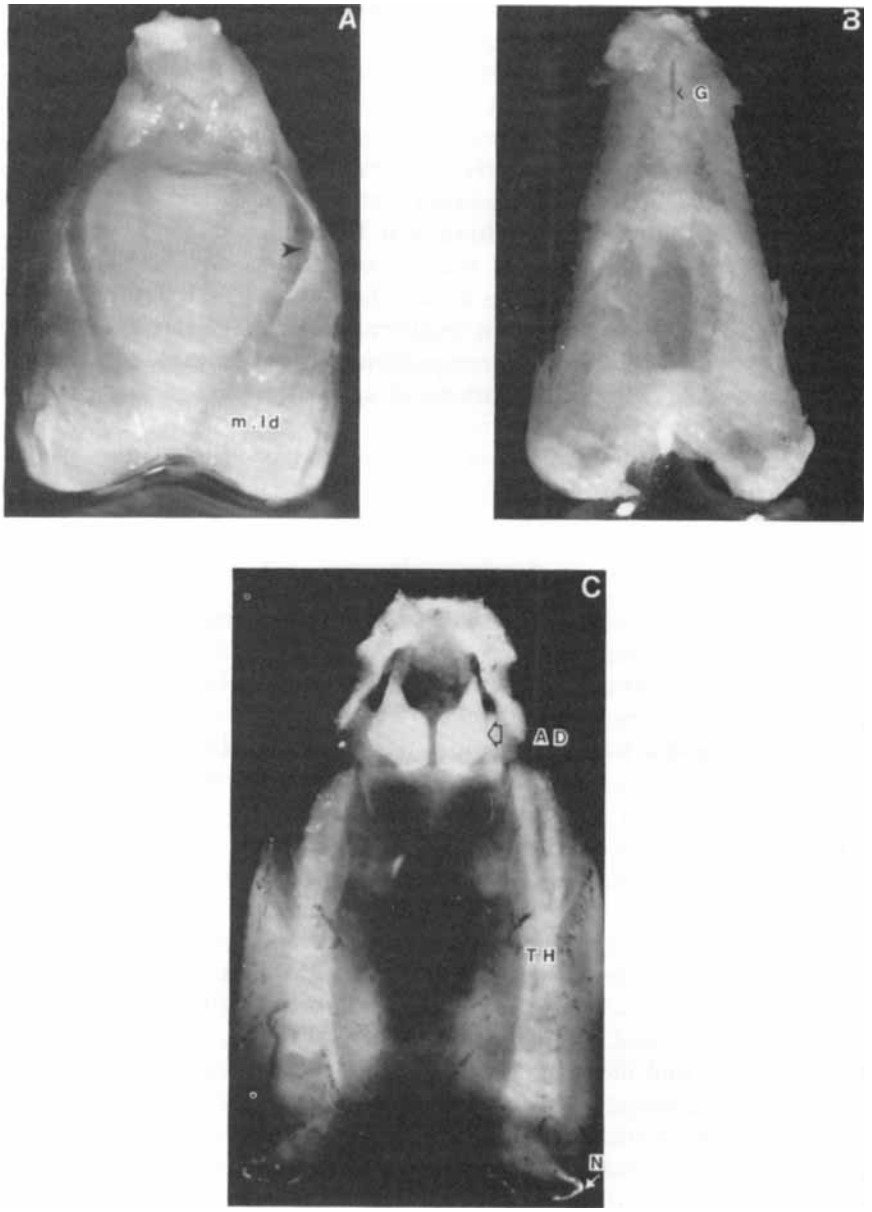


Fig. 3. The larynx of *Xenopus laevis* in dorsal view; anterior is up. (A) The larynx of an adult male, average weight 0.42g. The laryngeal dilator muscle (m.ld.) is indicated. The muscle is bipennate (arrowhead); a tendon connects with the arytenoid discs (see (C) below). (B) The larynx of an adult female, average weight 0.13g. Note the reduced muscle and cartilage mass, relative to the male. The position of the glottis (G), immediately dorsal to the arytenoids, is indicated. (C) The larynx of an adult male. The tissue was fixed in paraformaldehyde, dehydrated and cleared in methyl salicylate. Several internal cartilages including the arytenoid discs (AD) and thyrohyrals (TH) are apparent. Sound (individual clicks) is produced by movement of the arytenoid discs resulting from contraction of the *m. dilator laryngis* following activity on the laryngeal nerve (N).

Tobias and Kelley (1985) investigated the physiological mechanisms underlying vocal production. The larynx was removed and studied *in vitro*. When the laryngeal nerves of males were stimulated in a temporal pattern mimicking the mate call (i.e., 14 ms interstimulus interval for the fast portion of the call), a trill was produced with acoustic properties matching those of an actual mate call. Similarly, a call resembling ticking was produced when the nerves attached to an isolated female or male larynx were stimulated at long ISIs (>150 ms). There is a 1:1 correspondence between a muscle compound action potential, muscle contraction, development of tension and production of an audible click. Thus we suggest that vocal production in *X. laevis* results from a temporal pattern of activity of the laryngeal nerve which produces a corresponding series of muscle contractions and resultant clicks. Since the pattern of activity in the laryngeal nerve reflects the activity of laryngeal motor neurons, rapid firing leads to the rapid trilling of male mate call and slower discharge results in the slower trills of ticking.

THE NEURAL PATHWAY FOR VOCALIZATION

The laryngeal nerve contains axons of glottal and laryngeal motor neurons that exit the brain stem in the most caudal nerve rootlet of cranial nerve IX-X (Kelley, 1980; Wetzel et al., 1985; Tobias and Kelley, 1985; Simpson et al., 1986). The corresponding motor neurons are located in a slender column in the caudal medulla (cranial nerve nucleus IX-X, n. IX-X), just rostral to the spinal motor columns. Most laryngeal motor neurons are located laterally among the longitudinal fiber tracts; the glottal motor neurons occupy the rostral pole of n. IX-X. The dendrites of laryngeal motor neurons are directed medially, towards the adjacent inferior reticular formation, and also ramify anteriorly-posteriorly within n. IX-X itself. Horseradish peroxidase retrograde tracing studies reveal that n. IX-X neurons receive afferent input from inferior and medius reticular nuclei and from a small nucleus ventral to the cerebellum, the pretrigeminal nucleus of the dorsal tegmental area of the medulla (DTAM). DTAM is, in turn, innervated by telencephalic and diencephalic nuclei: the ventrolateral striatum, the preoptic area and postero-central and ventral thalamus. After a small injection of HRP-WGA into DTAM, many motor neurons are heavily labelled suggesting that laryngeal motor neurons send a recurrent collateral to DTAM, their major afferent source (Wetzel et al., 1985). The major connections that comprise the "calling circuit" are diagrammed in Fig. 4. The larynx is, as far as we know, used only in vocal behavior. Thus the connections uncovered using anatomical methods are likely to be involved in vocal production in *X. laevis* as are certain homologous nuclei in *Rana pipiens* (Schmidt, 1976; 1984). It is not yet clear how these brain regions function to produce the different temporal patterns required for ticking and mate calling.

SEXUAL DIMORPHISMS OF VOCAL NEUROEFFECTORS

Both the CNS vocal pathways and the larynx itself are highly sexually dimorphic. The projection from the preoptic area to DTAM and the recurrent collaterals from laryngeal motor neurons to DTAM are absent or greatly reduced in females (see Fig. 4; Wetzel et al., 1985). Laryngeal motor neurons

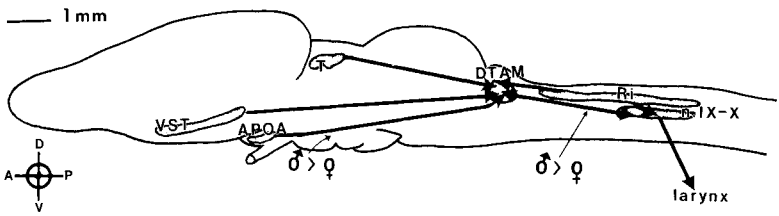


Fig. 4. Afferents to laryngeal motor neurons (n.1X-X) and the pretrigeminal nucleus of the dorsal tegmental area of the medulla (DTAM). Nucleus IX-X receives both ipsi- and contralateral input from inferior (Ri) and medius reticular nuclei as well as DTAM. Nucleus IX-X also sends a recurrent projection to DTAM. Nucleus DTAM receives ipsilateral input from ventral striatum (VST), anterior preoptic area (APOA) and several thalamic nuclei (T). The connection from APOA and n.IX-X to DTAM is less robust in females than in males. Data from Wetzel et al., 1985. Neurons in the VST concentrate estradiol (Morrell et al., 1975). Neurons in the APOA are also estradiol-concentrating; unlike the VST they are labelled after testosterone administration due to presumed aromatase activity (Morrell et al., 1975; Kelley et al., 1975; Kelley and Szmauz, unpublished). Several nuclei of the thalamus are labelled after either estrogen or androgen administration (Kelley et al., 1975; Morrell et al., 1975; Kelley, 1981). Nucleus DTAM, the RI and n.1X-X concentrate androgen exclusively. The larynx is also an androgen target (Segil et al., 1983; 1986).

are more numerous with larger somata and longer dendrites in males (Hannigan and Kelley, 1981; Kelley and Fenstemaker, 1983; Simpson et al., 1985). The larynx of males is 2–3 times the size of the female (Fig. 3; Ridewood, 1898; Segil et al., 1986). This sex difference is due to a larger muscle and cartilage mass in males. Males have an average of 32,000 muscle fibers (16,000 per side) while females have only 4000 fibers (2000 per side; Sassoon and Kelley, 1986).

Laryngeal muscle also displays a pronounced physiological dimorphism. Histochemical analyses reveal that male muscle is made up predominantly of one fiber type, most probably fast-twitch, fatigue-resistant, while female muscle is heterogeneous with the predominant type a slow-twitch fiber (Gray et al., 1985; Sassoon et al., 1986b). In recordings from isolated larynges of males (Tobias and Kelley, 1985), we found that discrete, large amplitude tension transients (sufficient to produce clicks) followed muscle compound action potentials down to interstimulus intervals as low as 7 ms. In females, however, such transients could only be produced at ISIs greater than 35 ms. When the laryngeal nerve of females is stimulated at the male mate call rate (14 ms ISI) only one click is produced at the onset of the stimulus train. Since successive stimuli within a train act, in females, to produce a maintained or tonic tension on the arytenoid discs (due to muscle tetany), we suggest that in response to repeated nerve stimulation the muscle in females remains contracted, the discs cannot relax to an apposed position, and clicks cannot be produced until stimulation ceases or occurs at a slower rate.

The pronounced laryngeal and brain sex differences of the vocal effector system suggest that the vocal capabilities of males and females are regulated both centrally and peripherally. One possibility is that the sexual differentiation of the central nervous system ensures that the temporal pattern mate calling is not produced in females. The characteristics of the muscle and, perhaps, neuromuscular junction may also ensure that the periphery

in females cannot respond. Female *X. laevis* have moderate levels of circulating androgen which increase in response to gonadotropin secretion (Lambdin and Kelley, unpublished). Sexual differentiation leading to a relatively androgen insensitive CNS and periphery may "protect" females from exhibiting inappropriate behaviors in response to androgen surges at ovulation.

ANDROGEN REGULATION OF MATE CALLING AND ITS NEUROEFFECTORS

The production of mate calling in adults and the developmental masculinization of calling neuroeffectors depend upon the secretion of gonadal androgens. Castration of adult males abolishes mate calling; the behavior is restored by treatment with testosterone or dihydrotestosterone but not estradiol (Wetzel and Kelley, 1983). Female *X. laevis* do not mate call. Since circulating levels of androgen are lower in females (Kelley, 1980; Lambdin and Kelley, unpublished), we administered androgen and attempted to evoke calling. Even protracted treatment with androgen failed to evoke a masculine call (Hannigan and Kelley, 1986). Many females did not vocalize at all, some displayed rapid ticking, and a few gave extremely brief, abnormally slow mate calls (Fig. 5). These studies suggested that some aspect of central or peripheral vocal production in the female constrains the ability to produce the very rapid trills of mate calling.

The larynx itself is an important contributor to constraints on female vocalization. As described above, the adult female can only produce clicks at ICI rates of 35 ms or longer (Tobias and Kelley, 1985). This limitation is probably due to metabolic/excitation-contraction properties of individual muscle fibers and/or to synaptic constraints at the muscle. Androgen treatment does not remove these constraints in adult females. While the andro-

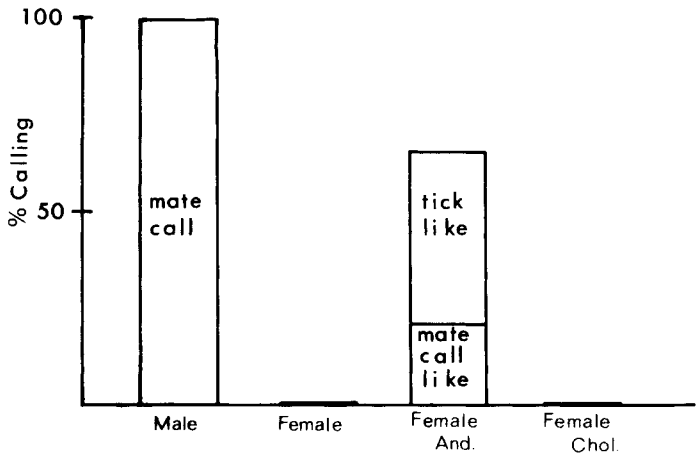


Fig. 5. Effect of androgen on vocalizations of female *Xenopus laevis* (data from Hannigan and Kelley, 1986). Under conditions which would have elicited prolonged bouts of mate calling from males, many females (approx. 30%) do not vocalize at all. Most females give brief, poorly modulated trains resembling ticking (tick-like vocalizations). A few females give very slow calls with fast and slow trill portions (mate call-like vocalizations). The *maximum* time spent vocalizing by any androgen treated adult female was 4.5 min in a 1-1/2 hour recording; the *average* time spent vocalizing by males was 45 min.

gen-treated female muscle can now follow shorter ISIs (down to an ISI of 21 ms) with discrete twitches, audible clicks cannot be produced at male-like stimulus frequencies since the continued presence of maintained muscle contractions hold the arytenoid discs apart. Some part of this physiological response is probably due to the properties of individual muscle fiber types in the female larynx. Male fibers are homogeneously fast-twitch and fatigue-resistant (Gray et al., 1985; Sassoon et al., 1986b). Females have a small number of male-like fibers, but also exhibit two other fiber varieties, small and large diameter, one of which probably accounts for the maintained muscle contraction to rapid stimulation that holds the arytenoid discs apart. Androgen treatment of adult females does not abolish maintained contraction and does not eliminate the two female-specific fiber types. Our recent experiments suggest that the failure of androgen to effectively masculinize adult females results from male-specific, androgen-regulated sexual differentiation.

ANDROGEN REGULATION OF LARYNGEAL DIFFERENTIATION

Our studies on the development of the larynx indicate that, at metamorphosis, the larynx is sexually monomorphic and feminine. Masculinization proceeds by alterations to the existing feminine phenotype. At metamorphosis the male and female larynx each have approximately 4000 muscle fibers (Sassoon and Kelley, 1986). There is little or no net increase in fiber number in the female between metamorphosis and sexual maturity. Males, on the other hand, show rapid rates of muscle fiber addition during the first year of postmetamorphic development (averaging 80 fibers/day) until the adult value of 32,000 fibers is attained. Accompanying this growth in fiber number is a concommitant growth in the laryngeal cartilages. Growth proceeds both by hyperplasia and hypertrophy. Ongoing rates of cell proliferation are high and muscle fibers increase in diameter during this period of growth. The process of sexual differentiation may be already in progress at metamorphosis since the rate of proliferation in males is greater than that of females at this time (Sassoon and Kelley, 1986).

The most probable mechanism for masculine differentiation of laryngeal muscle is androgen-induced myogenesis (Sassoon et al., 1983; 1986a). Administration of either testosterone or dihydrotestosterone to 3 week postmetamorphic juveniles of either sex results in a marked stimulation of mitoses in myoblasts and chondroblasts of the larynx specifically (Fig. 6). Like all other aspects of laryngeal neuroeffector function examined to date, cell proliferation is not induced by estradiol indicating that the response is androgen-specific. In adults, androgen does not induce myogenesis suggesting that hormone regulation of muscle cell proliferation is confined to sexually immature animals. The myoblasts that are stimulated to divide by androgen later appear as muscle nuclei in maturing fibers. In light of the pronounced sex difference in muscle fiber number of males and females, we believe that androgen secretion is responsibe for the rapid addition of new fibers to the larynx during the first year. Treatment with the antiandrogen, flutamide, prevents masculinization of fiber number (Sassoon and Kelley, 1986). Lack of the appropriate pattern of androgen secretion in females would thus account for the lack of muscle growth relative to males.

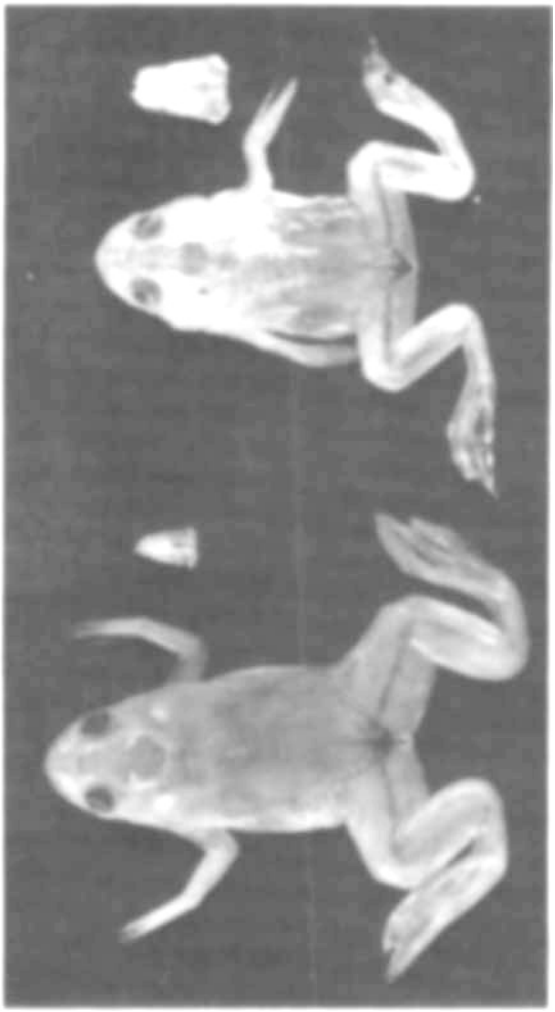


Fig. 6. Androgen induced laryngeal growth in juvenile female *Xenopus laevis*. Three week post-metamorphic frogs received testosterone propionate (right) or control (left) treatment for three weeks. Larynges are shown to the right of each animal; note the marked growth in the androgen treated female.

REGULATION OF THE ANDROGEN RECEPTOR IN LARYNX

The response of tissues to steroid hormones requires the presence of intracellular receptor proteins. We have examined the regulation of an androgen receptor protein in laryngeal muscle (Segil et al., 1983; 1986; Sassoon et al., 1985b). The *m. dilator laryngis* contains relatively high amounts of a specific, high affinity, saturable androgen receptor as compared to other muscles. The amount of receptor is two to three times higher in males than in females (Segil et al., 1983; 1986). At metamorphosis, levels of receptor are an order of magnitude higher than adult male values and are sexually monomorphic. Levels of androgen receptor in females begin to decline immediately following metamorphosis. In males, however, high levels of androgen receptor levels are maintained for the first 6 months and then fall to adult values (Sassoon et al., 1985b; Segil et al., 1986).

ANDROGEN LEVELS DURING DEVELOPMENT

These developmental changes (in morphology, in proliferation and in receptor levels) are paralleled by changes in blood levels of androgen (Lambdin and Kelley, unpublished). At metamorphosis, androgen levels are low and sexually monomorphic (~30 pg/mL). By 2–3 months, however, males have more than 3 times the circulating androgen levels of females and by 6 months, male levels are approximately 9 times those of females. Thus, the program of androgen secretion in the male includes moderate androgen titers (approx. 450 pg/mL) for the first 3–6 months after metamorphosis followed by an increase to high, adult males values (900 pg/mL) thereafter. Female values rise slowly and gradually during this period; by 10 months postmetamorphosis, titers are only 2/3 adult values (350 pg/mL). The first 6 months after metamorphosis correspond to a period of active myogenesis and high androgen receptor levels in males and little or no myogenesis with declining androgen receptor levels in females. Beginning at 6 months, high (adult-like) levels of androgen are achieved in males and receptor values begin to decline. We have found that exogenous androgen can down-regulate receptor levels in juvenile of both sexes; the active form of the hormone appears to be DHT (Sassoon et al., 1986c). Thus, endogenous secretion of gonadal androgens may contribute to the male decline in receptor amount seen between 6 and 10 months of development.

A MODEL FOR LARYNGEAL SEXUAL DIFFERENTIATION

Our observations on the development of the larynx can be related if we assume that the program of myogenesis in this tissue is sexually differentiated by male-specific patterns of androgen secretion. One possibility is that the myoblasts themselves contain high levels of androgen receptor. High receptor levels may enable males to begin proliferating in response to slowly increasing androgen secretion during the first two or three months. As long as myoblasts proliferate, high levels of receptor are expressed. As myoblasts start to fuse with each other to form new muscle fibers, however, androgen receptors down-regulate, accounting for the loss of androgen receptor that occurs as new muscle fibers are added to the larynx. Juvenile

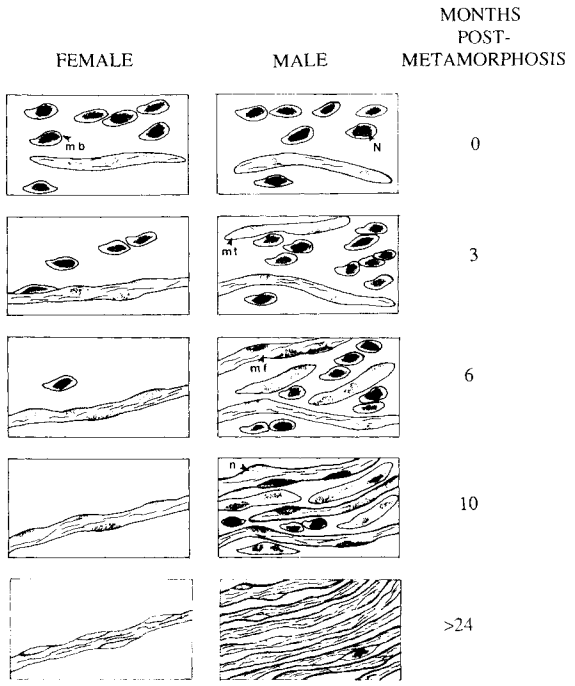


Fig. 7. A model for the sexual differentiation of laryngeal muscle in *Xenopus laevis*. At metamorphosis, the larynx is sexually monomorphic and feminine. Males and females have equivalent numbers of myoblasts (mb) and myofibers (mf). Levels of androgen receptor are high in myoblast nuclei (dark stipple) but down-regulate when myoblasts fuse to form myotubes (mt). At 3 months PM, male myoblasts are proliferating in response to moderate levels of androgen. Moderate levels of androgen secretion favor myoblast proliferation over myoblast fusion. Female myoblasts do not proliferate because androgen levels are low. Myoblast numbers in females decline due to cell loss and/or fusion with existing myofibers. By 6 months PM, males have reached high, adult-like levels of androgen secretion. These high levels favor myoblast fusion over proliferation (see 10 month panel) and androgen receptor levels begin to decline. By the time sexual maturity is well established, males have approximately 8 times the adult female number of muscle fibers. Few androgen receptor-rich myoblasts are present.

males, exposed to rising androgen titers during the first 6 months after metamorphosis, display continued myoblast proliferation and maintenance of high androgen receptor levels during this time. Juvenile females, without such exposure to androgen, gradually lose myoblasts (by fusion or by cell loss) and thus display a gradual loss of receptor during this period. A model outlining this process is diagrammed in Fig. 7.

Preliminary *in vitro* data suggest that stimulation of myoblast proliferation occurs at moderate levels of androgen secretion and that very high levels promote, instead, myoblast fusion (D. Sassoon, unpublished). We note that treatment with large amounts of androgen depletes the number of myoblasts in muscle and that the number of myoblasts that can be liberated, in culture, is less in 6 months old females than in males. Thus the moderate levels of androgen secreted during the first 5 postmetamorphic months in males could promote myoblast proliferation over fusion. The increase to adult values that is noted beginning at 6 months might promote myoblast

fusion over proliferation. While the line of thinking described above is congruent with the data we have collected to date, we caution that several features of the model have yet to be put to a direct test. It will, for example, be particularly important to determine the levels of receptor present in myoblasts as opposed to myotubes and mature muscle fibers.

SEXUAL DIFFERENTIATION OF THE CENTRAL NERVOUS SYSTEM; ONSET OF ANDROGEN SENSITIVITY

The neural "circuitry" for vocal production is markedly sexually dimorphic in adulthood. Males have approximately 1220 (610 per side) and females 740 (380 per side) motor neurons in studies of Nissl stained sections (Hannigan and Kelley, 1981). The mean size of the laryngeal motor neuron cell body is larger in males; males have more large neurons and females more small ones (Hannigan and Kelley, 1983; Simpson et al., 1986). The dendritic tree of male laryngeal motor neurons is much greater in extent (up to 270%) than that of females (Kelley and Fenstemaker, 1983). Neuroanatomical tracing studies suggest two differences in connectivity of these neurons: the projection from the preoptic area to DTAM is more robust in males than in females and the collateral from n. IX-X to DTAM is also a masculine characteristic (Fig. 3). We have used staining for the mitochondrial enzyme, succinic dehydrogenase, to demonstrate that DTAM is also dimorphic in *X. laevis* (Haerter and Kelley, unpublished), a finding confirming that of previous studies in other anurans by Schmidt (1983; 1984). These efferent nuclei are the most accessible to study and we do not rule out further sex differences in neuronal morphology or circuitry (including afferents to vocal control regions) as these connections are studied in further detail.

We do not yet know when, during development, sex differences in cell number, dendritic extent or connectivity are established. Some of these dimorphic characteristics are sensitive to androgen in adulthood and some are not. Dendritic extent of adult female laryngeal motor neurons is not increased by androgen (Shih and Kelley, unpublished), nor is the number of motor neurons in adulthood (Hannigan and Kelley, 1983). If androgen regulates these dimorphic features, it must act at an earlier developmental stage (as it does, for example in the rat SNB system; Breedlove, 1986). The somal size of adult females can be shifted in the masculine direction by androgen although a completely male-like distribution is not achieved (Hannigan and Kelley, 1983). The somal size of juveniles (immediately pre- and postmetamorphic) is increased by androgen in both sexes suggesting that hormone sensitivity is already present before metamorphosis.

In the adult, many of the brain nuclei that effect vocal behaviors include androgen-concentrating neurons (Kelley et al., 1975; Kelley, 1980; 1981). Many cells in DTAM, most of the laryngeal motor neurons, and cells in the thalamus, reticular formation and preoptic area are labelled after 3H-testosterone or 3H-DHT administration. We determined the onset of androgen sensitivity in these neurons in an autoradiographic study (Gorlick and Kelley, 1986). At metamorphosis, the main features of the adult labelling pattern are already established although some nuclei contain fewer labelled cells than in the adult (and one, a vestibular nucleus, has no labelled cells). Administration of 3H-DHT to stage 60 tadpoles yields no labelling while

administration at tadpole stage 64 reveals extensive labelling. We thus believe that CNS androgen sensitivity, as measured by the presence of autoradiographically demonstrable androgen accumulating neurons, develops between stages 60 and 64, a few weeks before metamorphosis.

One major difference between the androgen sensitivity of larynx and CNS is that the former is sexually dimorphic (at all stages examined beginning with 3 months PM), while the latter is not. The adult male larynx has 2 times the female level of receptor (Segil et al. 1983; 1986). The distribution, number, and extent of labelling in androgen cells in the adult central nervous system is not markedly dimorphic (Kelley et al., 1975; Kelley, 1981). Another apparent difference is the time course over which the adult pattern of receptor expression is achieved. In larynx, levels of receptor start high (just after metamorphosis) and achieve adult values by a gradual decrement over a 10 month period. In CNS, an essentially adult pattern appears rapidly during the two weeks preceding metamorphosis. The relation between androgen responsiveness and proliferation also differs for the two tissues. In larynx, receptor values are very high during the period of intense cell proliferation and decline, to adult values, thereafter. In brain, the adult pattern of DHT concentrating neurons is already present at metamorphosis, but vocal neurons have ceased to proliferate (Gorlick and Kelley, 1984; 1986 and in preparation). In fact, most neurons of the vocal effector circuit are postmitotic before the gonads are capable of androgen secretion. Unlike the larynx, androgen probably does not regulate proliferation of CNS target cells; we observe no sex difference in rate of proliferation when developing frogs from embryonic Stage 11 (Nieuwkoop and Faber, 1954) to 3 week postmetamorphosis are examined (Gorlick and Kelley, 1984 and in preparation). The best candidate mechanism for regulating laryngeal motor neuron number is androgenic prevention of cell death (see Nordeen et al., 1985).

SITE OF ANDROGEN ACTION: CNS AND/OR PERIPHERY

Masculinization requires the expression of a functional androgen receptor protein by hormone target cells. Administration of androgen to animals either during developmental "critical periods" or in adulthood results in altered morphology and function of target neurons. Since hormone-sensitive neurons are extensively interconnected (including projections of motor neurons to androgen-sensitive muscle), it has never been clear whether effects of androgen are due to direct hormone action on target cells and/or to indirect effects exerted via anterograde or retrograde synaptic influences. Toran-Allerand (1976; 1984) has documented the very powerful effects of estrogen (a masculinizing hormone in rodents) on neurite outgrowth of hypothalamic explant cultures and has proposed that the process of masculinization might begin at the hypothalamus and then extend, via the extensive synaptic connections of these neurons, anterogradely to all portions of the "circuitry" for reproductive function.

An alternative hypothesis, for sexually dimorphic neuromuscular systems such as frog larynx and the rat SNB, is that the process of masculinization begins at the periphery and extends, retrogradely, back into the central nervous system permitting and stimulating the sexual differentiation of neurons that participate in male-specific behaviors. In this view, masculinization of the central nervous system begins at the muscle and is best ex-

emplified by work on the levator ani/bulbocavernosus complex in rats (Breedlove, 1986) and studies of the frog larynx (see Introduction).

How might androgen act at the muscle to prevent motor neuron cell death? It is reasonable to suspect that, in target muscles, androgen regulates one or more of the major processes implicated in neuron cell death: size of the muscle target and muscle activity (Hamburger, 1975; Pittman and Oppenheim, 1979). In frog larynx, androgen controls the process of myogenesis and is responsible for the addition of new muscle fibers in males during postmetamorphic development (Sassoon et al., 1986a; Sassoon and Kelley, 1986). An increase in the size of the periphery has been shown, in many systems, to reduce ontogenetic cell death of appropriate motor neurons. In *X. laevis,* the male adds new muscle fibers at an average rate of 80/day during the first year (Sassoon and Kelley, 1986). There is no net addition of fibers in the female. Thus, the male is confronted with a continually expanding periphery and motor unit size should continue to grow during the first postmetamorphic year. A reduction in motor unit size is associated with the process of synapse elimination in many systems (Brown et al., 1976; Dennis et al., 1981). Our data indicate that the male larynx provides a much greater synaptic field than does the female larynx, a factor which could contribute significantly to the expression of sexual dimorphism in laryngeal motor neurons.

Ontogenetic cell death can be prevented by eliminating muscle activity (Pittman and Oppenheim, 1979). If muscle fibers are prevented from depolarizing and contracting, more synapses per fiber are maintained and more motor neurons are supported. Androgen sensitivity has been shown to be associated with a prolongation of polyinnervation in rat levator ani (Jordan and Arnold, 1985). We propose that this effect occurs because androgen blocks depolarization of muscle fibers or muscle contraction during the developmental critical period. While there is, as yet, no direct evidence for this hypothesis, androgens have a variety of powerful effects on acetylcholine receptor channels of *X. laevis* laryngeal myotubes (Erulkar and Wetzel, 1985), which could contribute to changes at the level of the muscle membrane. Androgens could also affect the contractile proteins of the fiber, perhaps maintaining an embryonic or less functional form during this developmental period as has been proposed for embryonic forms of tropomyosin (Ordahl, personal communication). Either effect could reduce muscle contraction and help to maintain sufficient levels of a trophic factor (motor neuron growth factor) such that muscle fibers support more synapses and/or motor neurons.

Thus we propose that the first step in the masculinization of many sexually dimorphic neuromuscular systems is androgen action on the muscle which permits survival of the motor neurons. A second step could be androgen-induced dendritic growth. Androgen receptor is present in laryngeal motor neurons themselves and in afferents (see Fig. 4). Toran-Allerand's work suggests that a major effect of steroids on developing CNS is the elaboration of extensive dendritic trees (1976; 1984). Male laryngeal motor neurons have much more extensive dendritic fields than those of females (Fenstemaker and Kelley, 1983; Simpson et al., 1986). The length of each dendritic branch is longer in males and the cumulative result is that the male neuron can have up to 270% times the extent of the female. The production and maintenance of this arbor may require androgen action on the motor neuron itself

and also require input from androgen sensitive afferents. A role for synaptic afferents in stimulating dendritic growth of certain cells has been reported (Globus and Scheibel, 1967; Valverde, 1976).

ANDROGEN-DIRECTED SYNAPSE FORMATION

Recent evidence (Konishi and Akutagawa, 1985; Wetzel et al., 1985) suggests that the connections of vocal control nuclei in the male brain may be quite different from those of the female. In zebra finches, the projection from the telencephalic vocal control nucleus HVc to nucleus RA is incomplete in females; afferents end in the lamina overlying RA and do not penetrate into RA itself. In clawed frogs, at least two connections are absent or reduced in the female—the projection from preoptic area to DTAM and the recurrent collateral from laryngeal motor neurons to DTAM (Fig. 4).

Findings in zebra finches and clawed frogs suggest that during the process of CNS sexual differentiation male-specific synaptic connections are formed, presumably under the influence of male-specific patterns of hormone secretion. One possibility is simply that females have fewer neurons with less robust axonal trajectories than males. This, combined with a less extensive dendritic arbor of target neurons, would serve to ensure that the female projection is a greatly attenuated and poorly functional version of the male. An alternative is that androgen allows the establishment of a favorable milieu for axonal navigation and male-specific synapse formation with appropriate targets.

FUTURE DIRECTIONS

Two major problem areas remain. One is the relation of the multiple sexual dimorphisms in the CNS and in the larynx to sex differences in behavior. We will require knowledge of how sex-specific vocal patterns, mate calling and ticking, are generated and how specific CNS dimorphisms—cell number, dendritic length and connectivity—contribute to behavioral differences. The second problem is to determine how androgenic steroids harness the developmental programs of muscle and nervous system and effect differentiation. At the cellular level, we need a more complete description of androgen effects and to determine which are direct and which are indirect. At the molecular level, how does androgen receptor occupation induce one cell type (myoblasts) to proliferate and another (motor neurons) to add postsynaptic membranes? The advantages of an androgen-sensitive neuromuscular system will greatly facilitate progress on these issues.

I have been fortunate to work with many talented laboratory colleagues at Princeton and Columbia Universities and gratefully acknowledge the contributions of Suzanne Fenstemaker, Dennis Gorlick*, Grace Gray, Ursula Haerter, Patricia Hannigan, Laren Lambdin*, Linda Long, David Sassoon*, Neil Segil, Scott Shih, Martha Tobias*, Andrea Weintraub and Daniel Wetzel. I thank current members of the laboratory (*) for helpful comments on this review. The research described here was supported by grants NS 19949 and NS 23684 from the NIH, as well as an RCDA (HD 00493) and a Sloan Foundation Fellowship.

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