

The Vocal Motor Neurons of *Xenopus laevis*: Development of Sex Differences in Axon Number

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SUMMARY

Sex differences in the number of muscle fibers in the larynx of clawed frogs (*Xenopus laevis*) develop after metamorphosis. In order to examine possible contributions of neural innervation to this process, we prepared sections of the laryngeal nerve from tadpole stage 56, when the sexes can first be distinguished, through adulthood, and counted axons on electron micrographs. The adult number of axons is achieved by a sexually differentiated pattern of axonal addition and loss. Axon numbers are high at tadpole stage 56 and equal for males and females; thereafter, males have more axons. Sex differences are most pronounced at tadpole stage 62 because between stages 59 and 62 the number of laryngeal axons in males increases by an average of 119 per nerve. Ultrastructural evidence is congruent with the hypothesis that new axons are added to the laryngeal nerve between tad-

pole stages 56 and 62. The loss of axons from the laryngeal nerve is greater for females than for males. Between tadpole stages 56 and adulthood, overall axon number decreases by 47% in males and by 64% in females. Signs of axonal degeneration are present in both sexes before metamorphosis but are rare at juvenile or adult stages. The numbers of axons in juvenile frogs do not differ from those in adults and continue to be greater in males than in females. In contrast to the amount of axon addition and loss, the timing of axon loss and the percentage of myelinated axons is the same for males and females throughout development. Thus sex differences in the innervation of laryngeal muscle originate before metamorphosis and could contribute to the marked sex differences in muscle fiber addition that occur thereafter.

INTRODUCTION

Sex differences in cell number are common in neural and muscular systems specialized for reproductive functions (Kelley, 1988). In vertebrates, these sex differences are due to the secretion of gonadal steroids at particular times in development. Steroid hormones have been shown to increase cell proliferation in developing muscle (Sassoon, Segil, and Kelley, 1986) and to prevent ontogenetic cell death in brain (Konishi and Akutagawa, 1985; Nordeen, Nordeen, Sengelaub, and Arnold, 1985). Because affected cells express the appropriate steroid hormone receptor protein (Kelley, Sassoon, Segil, and Scudder, 1989; Gahr and Konishi, 1988), hormones could be exerting

direct control of target cell proliferation or cell death. However, both muscle and brain cells rely on synaptic partners during early development. Steroid effects could thus be indirect via action on steroid-responsive afferent neurons or synaptic targets. These issues are difficult to resolve within the central nervous system due to its synaptic complexity. Sexually dimorphic neuromuscular systems provide more tractable experimental preparations (Kelley, 1986; Kelley, 1988), especially since mechanisms controlling the number of motor neurons and muscle fibers have been extensively documented in systems that are not steroid-sensitive (Oppenheim, 1985; Stockdale, 1982).

In the clawed frog, *Xenopus laevis*, the neuromuscular system that controls courtship song becomes sexually differentiated under the influence of testicular androgen secretion (Kelley and Tobias, 1989). Sounds are produced by laryngeal muscle contractions in response to activity in laryngeal motor neurons (Tobias and Kelley, 1987). Adult males have more laryngeal muscle fibers

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(Sassoon and Kelley, 1986) and neurons in the laryngeal motor nucleus (Hannigan and Kelley, 1981). At metamorphosis, when the tadpole transforms into an immature frog, the number of laryngeal muscle fibers is the same for males and females. The male frog then adds additional muscle fibers at a rapid rate (150 fibers/day) for the next 6 months; the rate of muscle fiber addition in females is much slower (25 fibers/day; Marin, Tobias, and Kelley, 1990). The male rate of muscle fiber addition depends on testicular androgen secretion (Sassoon, Segil, and Kelley, 1986; Sassoon and Kelley, 1986; Marin et al., 1990). Both laryngeal muscle and motor neurons express androgen receptor during the period of postmetamorphic muscle fiber addition (Kelley, Sassoon, Segil, and Scudder, 1989; Gorlick and Kelley, 1986). Are the effects of androgen on muscle fiber addition exerted at the level of laryngeal muscle (direct effects) or at the level of the laryngeal motor neurons (indirect effects)?

Motor neurons rely on muscle targets for survival (Oppenheim, 1985). In another sexually dimorphic system (rat perineal muscles and their motor neurons), the larger number of motor neurons in males is due to androgen rescue of muscular targets that would otherwise have degenerated (Fishman and Breedlove, 1988; Fishman, Chism, Firestone, and Breedlove, 1990). Sex differences in SNB motor neuron numbers follow sex differences in perineal muscles. Many muscles, however, will not produce a normal number of muscle fibers unless they are first innervated (Stockdale, 1982). Thus sex differences in motor neuron number could control muscle fiber number rather than vice versa. If androgen acts at the level of laryngeal muscle to control laryngeal motor neuron number in *X. laevis*, then sex differences in neural innervation should follow the establishment of sex differences in muscle fiber number. If, on the other hand, androgen acts at the level of the motor neuron, then sex differences in innervation of laryngeal muscle should precede sexual differentiation of muscle fiber number. The present study attempts to distinguish between these possibilities by determining when, relative to sexual differentiation of muscle fiber number, sex differences in laryngeal axon number arise.

MATERIALS AND METHODS

Axon Counts

Animals. Adult (2 years postmetamorphic (PM), 40–83 g body weight: 5 male, 7 female) and juvenile (3–6 months postmetamorphic, 9–10 g body weight: 3

male, 3 female) frogs were obtained from *Xenopus I*, Ann Arbor, MI. Tadpoles at stage 66 (3 male, 4 female), stage 62 (4 male, 6 female), stage 59 (4 male, 4 female), and stage 56 (5 male, 3 female) were obtained from Nasco, Fort Atkinson, WI or were lab-bred. One animal each at stages 52 and 54 was also examined; these tadpoles could not be sexed because the gonads had not yet differentiated and sex chromosomes cannot be distinguished in karyotypes. Tadpoles were assigned to pre-metamorphic stages using the criteria of Nieuwkoop and Faber (1956), which rely on external morphological features (e.g., limb buds and tail length). At stage 66, frogs have just completed metamorphosis. Juveniles were at postmetamorphic stage 5 (average male laryngeal weight, 170 mg; average female laryngeal weight, 37 mg) using the criteria of Marin et al. (1990), which rely on laryngeal weight and muscle fiber number. Adults were at PM stage 6 (male laryngeal weight > 400 mg; female laryngeal weight > 150 mg).

Dissection. Each animal was deeply anesthetized by immersion in 0.1% MS222 (ethyl *m*-amino benzoate, methanesulfonic acid; Aldrich Chemical Co.) and the larynx exposed. Sexually immature and adult larynges were cleaned of surrounding muscle and other connective tissue, thus freeing the attached laryngeal nerve. The nerve was then cut at its point of entry into the muscle and pinned out for fixation. Tadpole larynges were dissected *in toto* and prepared for electron microscopic (EM) examination of the attached laryngeal nerve. Male and female nerves at a given stage were processed together.

Sex Determination. Adult and sexually immature animals were sexed by gonadal inspection using a Zeiss dissecting microscope. For tadpoles, the entire interrenal gland with attached gonads was removed and fixed in 1% glutaraldehyde for 1 h. The tissue was then paraffin-embedded, sectioned at 15 μ m and sections were stained with hematoxylin and eosin. Sections were compared to a normal series of developing male and female gonads (Witschi, 1971). Using sections prepared in this manner, males and females can be distinguished from stage 56 on.

Tissue Processing. Pieces of nerve or whole larynges were fixed in 1% glutaraldehyde (Sigma, grade 1) in phosphate buffer (PB) (0.1 M, pH 7.2) for 1 h, followed by a brief rinse in PB and postfixation in 1% osmium tetroxide (Sigma) in PB for 2 h. Then tissue was again rinsed in PB and dehydrated through a series of alcohols and in propylene oxide (Fisher).

Embedding. The dehydrated tissue was infiltrated with dilute (2:1, then 1:1) LX112 resin (by volume: 45.3% LX112, 30.5% DDSA, 22.7% NMA, 1.5% DMP; all from Ladd) in propylene oxide followed by overnight infiltration in fresh resin solution. The tissue was then

placed in resin within molds and hardened at 60°C for 48–72 h.

Electron Microscopy. Many axons are small ($<1 \mu^2$ in area), even in adults, and accurate counts required electron microscopy. Nerves from adult and juvenile frogs were sectioned close to their point of entry into the laryngeal muscle at the posterior pole of the larynx. To obtain thin sections of the younger nerves at a point before, or just after, entry into the muscle, and to most closely approximate the level at which the older nerves were cut, thick ($\sim 1.0 \mu\text{m}$), transverse sections were taken through each larynx from the anterior to the posterior pole until a point as close as possible to the nerve's exit from the laryngeal muscle. Thin sections for EM analysis were then obtained. Sections were cut at 80 nm on a Sorvall MT 5000 with a Diatome diamond knife and were examined on 50 mesh Formvar-coated copper grids (stages 52–66) or $1 \times 0.5 \text{ mm}$ Formvar-coated copper slot grids (postmetamorphosis) using a JEOL 1200EX transmission electron microscope. Photographs were taken of the nerves at 400–500 \times for postmetamorphic frogs and at 6000–15,000 \times for animals at earlier stages in development. Adults and sexually immature frogs were also examined at 2,500 \times to ensure that no small caliber axons were missed. Photographs were then assembled into montages of entire nerve sections for axon counts.

Axon Counts. Axons were identified as membrance-bound profiles containing microtubules and neurofilaments. Glial processes were distinguished from axons by cytoplasm containing rough endoplasmic reticulum. The laryngeal nerve of adults contains axons of laryngeal motor neurons and axons of glottal motor neurons (Simpson, Tobias, and Kelley, 1986); there are no muscle spindles in the larynx (Sassoon et al., 1986). Counts of axons in the laryngeal nerve thus represent numbers of motor axons. Since in preliminary studies of three or four sections of adult laryngeal nerves, counting error was less than 5%, we confined counts of each nerve to a single section. Left and right laryngeal nerves were taken from two adult male and two adult female frogs, and axon counts were compared. There was no significant difference between the number of axons in the left (L) or right (R) laryngeal nerves of adults (L: 249 ± 5.6 ; R: 243 ± 5.2). The section from the left or right laryngeal nerve with the best preservation of ultrastructure was thus chosen and the total number of axons was counted on photographic montages. All axon counts are reported as means \pm standard deviations. The significance of differences in total axon number was evaluated using analysis of variance (Statview; Brain Power Inc.) with post hoc *F*-tests (Sokal and Rohlf, 1981). Differences between left and right nerve axon numbers and between male and female sciatic nerve axon numbers were evaluated with Student's *t*-test. Two-tailed tests were used in all cases.

Controls. Sciatic nerves were used as controls to determine whether sexual dimorphism in axon number is a feature of other *X. laevis* nerves. Left and right sciatic nerves were dissected from three adult male and three adult female frogs immediately after removal of the laryngeal nerves and were processed in an identical manner. There was no statistically significant difference between the number of axons in the left or right sciatic nerve of adults (L: 2933 ± 346 ; R: 2798 ± 294 , $p > 0.05$). Sex differences in numbers of axons in the sciatic nerve were not statistically significant (*F*: 3096 ± 261 ; *M*: 2686 ± 266 , $p > 0.05$).

RESULTS

Axon Counts

Axon counts from developing laryngeal nerves are presented in Figure 1. In both sexes, axon number rises between tadpole stages 52 and 56 and then falls, reaching adult values sometime between metamorphosis (stage 66) and the juvenile stage. At tadpole stage 56, axon counts are the same for males and females (individuals at stage 52 and 54 could not be sexed because the gonads have not yet differentiated). From stage 59 on males have, on average, more axons in the laryngeal nerve than females have. Male/female differences in axon numbers are most pronounced at tadpole stage 62.

The variability of axon counts is similar within premetamorphic groups and within postmetamorphic groups but not between groups. We thus analyzed statistical differences separately using an analysis of variance (ANOVA). Results of ANOVAs indicate significant contributions by sex ($p < 0.025$) and age ($p < 0.005$) within the tadpole group. After metamorphosis, sex alone was a significant factor ($p < 0.025$). Post hoc *F*-tests indicated that male/female differences in axon counts were significant at stage 62 ($p < 0.005$), juvenile ($p < 0.05$), and adult ($p < 0.001$). For female tadpoles, counts at stage 56 differed significantly from those at stage 62 and at stage 66 ($p < 0.01$). For male tadpoles, counts at stage 56 differed significantly from those at stages 62 and 66 ($p < 0.005$). Counts of axon numbers in the male laryngeal nerve at stage 62 were significantly different than counts at stage 66 ($p < 0.01$), but not at stage 59 ($p > 0.05$).

DESCRIPTION OF MALE AND FEMALE LARYNGEAL NERVES—METHODS

Two processes account for developmental changes in axon number in the laryngeal nerve: addition of

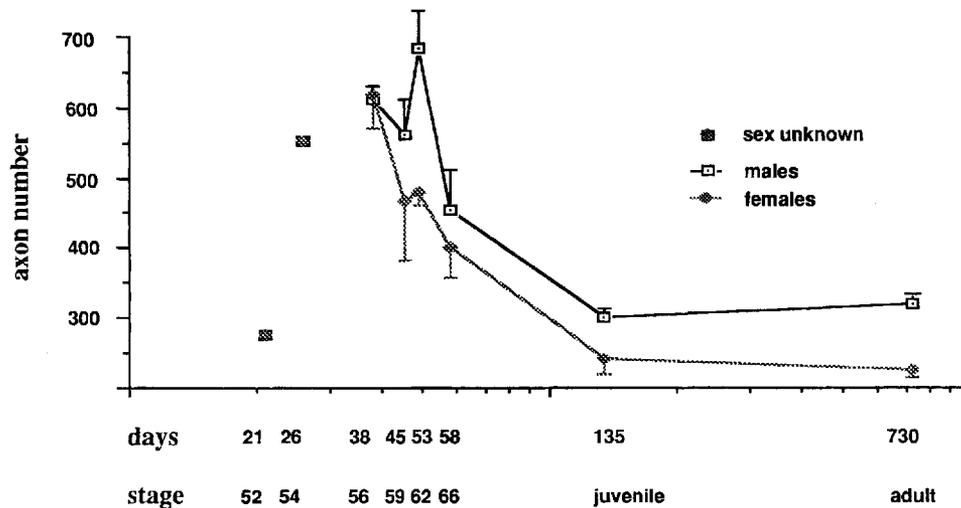


Figure 1 Changes in motor axon number in the developing laryngeal nerve. The mean number of axons in each laryngeal nerve from stage 56 through adulthood is shown as is the number of axons in each specimen at stages 52 and 54. Error bars indicate standard deviations. Axon number is the same for males and females at stage 56; males have more axons thereafter. Sex differences are significant at stages 59 ($p < .05$), 62 ($p < .001$), juvenile ($p < .005$), and in adulthood ($p < .001$). The sex difference in axon number at stage 66 is not significant ($0.1 > p > .05$).

new axons and loss of existing axons. Some new axons must be added to the male nerve after stage 56 to account for the significantly larger number of axons seen at stage 62. Some existing axons must likewise be lost to account for the significant decrease seen, for example, in females between tadpole stages 56 and 62. We reasoned that axon addition might be accompanied by morphological signs of the presence of young axons (growth cone-like morphology, small size, unmyelinated) and axon loss might be accompanied by ultrastructural evidence of degenerating axoplasm. We thus examined the electron micrographs for these features (Fig. 2 and 3) and estimated the fraction of such axons at each stage for each sex.

Immature axons have growth cones at their tips. Growth cones include a central core region and a distal fringe. This distal portion of the growth cone contains lamellipodia. In thin sections, lamellipodia appear as extended, membrane-bound profiles containing microfilaments and occasional vesicles (cf. Williams, Bastiani, Lia, and Chalupa, 1986). For each section from which we had obtained total axon counts, we also identified and counted axons containing lamellipodia. We then further characterized the maturation of axons in these laryngeal nerve sections by analyzing their degree of myelination. Myelinated, myelinating, and unmyelinated axons (Fig. 2) were counted on each photo-

montage. We defined myelinating axons as those having at least 75% of the plasma membrane enveloped by a glial process (Fig. 2). To assist in our qualitative description of axonal maturation, we also took one section from a male and from a female nerve at each stage and measured (Sigma Scan, Jandel Scientific) the area of each axon. Finally, we examined the photomicrographs for the presence of axon profiles containing dark inclusions, dilated, and very electron-dense mitochondria or dense lamellar structures, and disrupted plasma membranes (Fig. 3). These characteristics have been interpreted as axonal necrosis arising from death of parent somata (Guillery, 1970; Chu-wang and Oppenheim, 1978; Williams et al., 1986). We recognize that axon loss could also be attributable to retraction. We do not know if signs of necrosis in axons are reliable ultrastructural correlates of retraction. Results of this survey of developing male and female laryngeal nerves are described below.

DEVELOPING MALE AND FEMALE LARYNGEAL NERVES—RESULTS

Growth of the Laryngeal Nerve and Its Axons

The nerves of specimens examined at stages 52 and 54 were very small ($<20 \mu$ in diameter). The

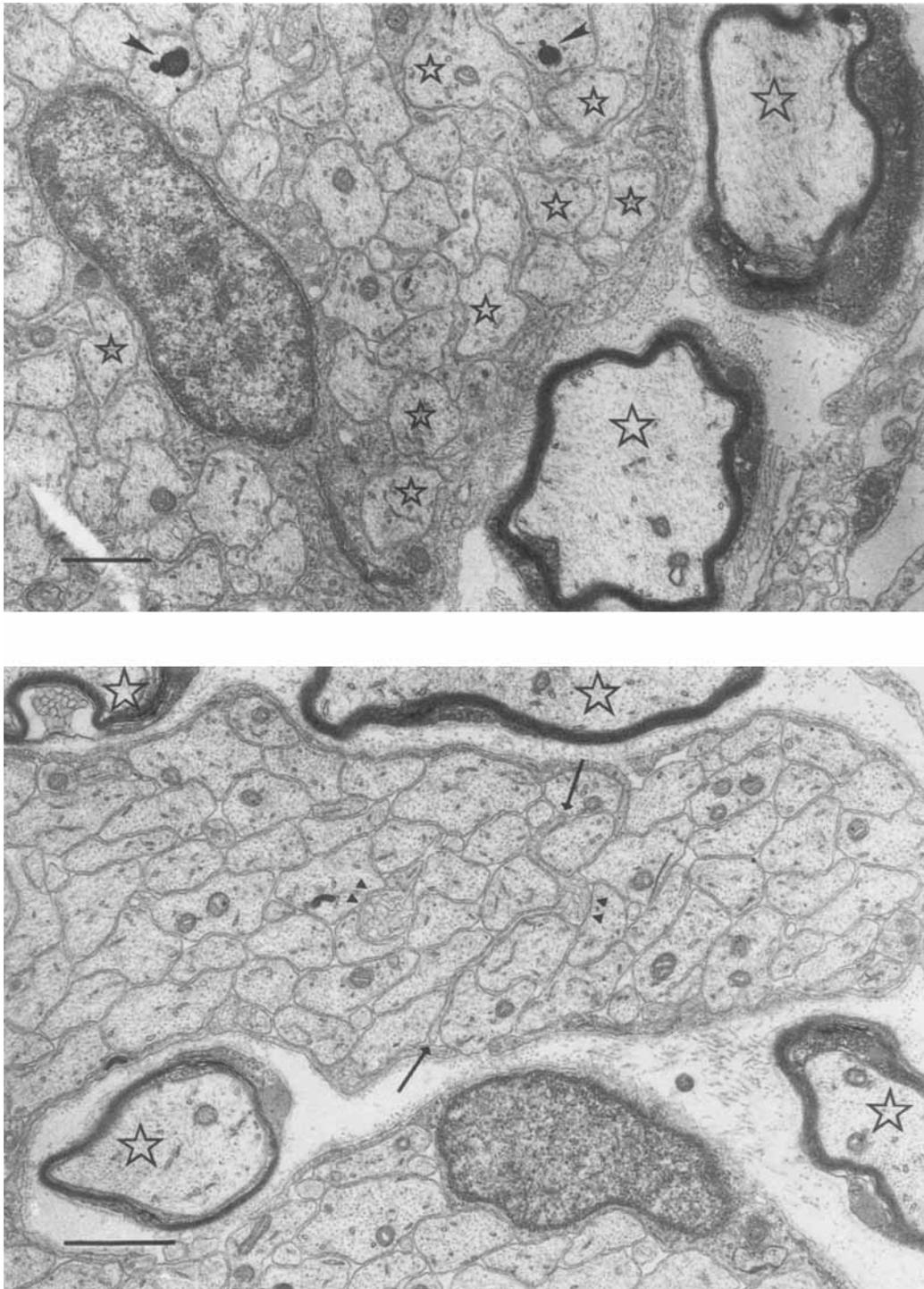


Figure 2 Electron micrographs of regions at the periphery of laryngeal nerves in *X. laevis* tadpoles. (Upper) Axons in the process of myelination (small stars) are surrounded by glial cytoplasm. Larger, myelinated axons (large stars) occupy the periphery of axon fascicles. Two axons contain electron-dense mitochondria (arrows) but do not appear disrupted. Male tadpole, stage 62; scale bar = 1 μ . (Lower) An extended axonal process (between two arrows), characteristic of growth cones, occupies the central area of the axon fascicle. This process includes a larger, vesiculated region (double arrowheads). Similar regions (at left) are frequently seen connected to the main body of the axon by a "neck." Processes of glia can be distinguished from axonal processes by the occurrence of rough endoplasmic reticulum in the former (eg, surrounding cell nucleus at lower right). Male tadpole stage 59; scale bar = 1 μ .

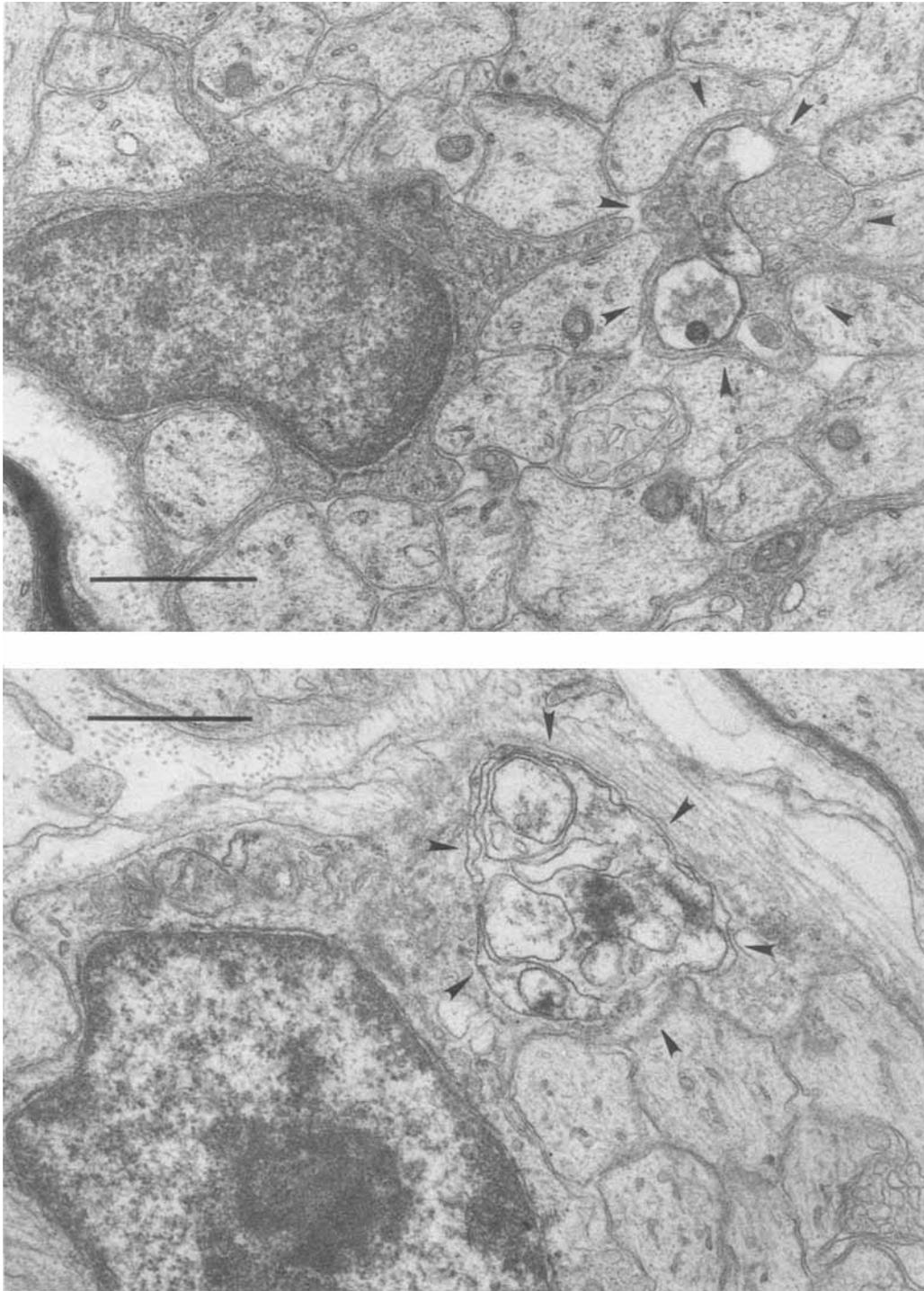


Figure 3 Electron micrographs of degenerating axons (surrounded by arrowheads) in the laryngeal nerves of two female stage 62 tadpoles. Several axonal profiles that include dark and mottled axoplasm and electron-dense structures (probably mitochondria) are surrounded by a glial process. Myelinated axons also appear to degenerate (not shown). Scale bars = 1 μ .

stage-52 nerve contained unmyelinated axons; most (65%) were small ($<1 \mu^2$ in area) but the nerve also contained large axons ($>5 \mu^2$ in area; 30% of the total axon number). The large axons were grouped together, separated by clusters of small axons. The nerve examined at stage 54 contained many small axons (90% $<1 \mu^2$ in area); the largest axons, located primarily at the periphery of the nerve, were myelinated.

The average size of axons increased slowly throughout premetamorphic development (from about $0.6 \mu^2$ in area at stage 56 to about $0.9 \mu^2$ in area at stage 66). By metamorphosis, the average size of axons in the laryngeal nerve of males was larger than that of the female (mean area for axons in a male nerve: $1.2 \mu^2$; range: $.05-9 \mu^2$; mean area for axons in a female nerve: $0.6 \mu^2$; range: $.01-6 \mu^2$). This larger axonal size in males, together with a greater number of axons, results in the overall gross sexual dimorphism of the laryngeal nerve observed from tadpole stage 66 (Fig. 4) through adulthood. Axons continue to enlarge after metamorphosis (average area about $8.5 \mu^2$ for juveniles and $15.5 \mu^2$ for adults).

Characteristics of Developing Axons— Varicosities and Growth Cones

Axonal profiles of developing laryngeal nerves in *X. laevis* largely resemble those seen in motor nerves of other species (e.g., chick; Chu-Wang and Oppenheim, 1978) and in sensory nerves (Williams, et al., 1986). A prominent feature of developing *X. laevis* laryngeal axons is the presence of axons containing large (7–18 nm), irregular vesicles (Fig. 2). Similar but smaller (2–11 nm in diameter) vesicles were occasionally observed within glial processes. Axons containing such vesicles were often irregular in profile; the portion containing the vesicles attached by a “neck” to the main body of the axon. Such axons were otherwise unremarkable; microtubules and neurofilaments did not appear disrupted. In developing chick ciliary ganglion axons (Landmesser and Pilar, 1976) and motor nerves (Chu-Wang and Oppenheim, 1978), similar vesicles have been described associated with axon varicosities. The incidence of large vesicle-containing axons was greatest during early stages (52–62); these profiles are absent following metamorphosis. No sex differences in the occurrence of vesicle-containing axons was apparent (stage 56: male, 4.0%; female, 2.1%; stage 59: male, 1.9%; female, 3.6%; stage 62: male, 2.0%;

female, 2.4%; and stage 66: male, 0.3%; female, 0.9%).

All nerves examined before tadpole stage 66 contained extended axonal profiles. These profiles (Fig. 2), resemble those identified by others (c.f. Williams et al., 1986) as the lamellipodial extensions of growth cones. The number of such profiles was small (1–3 per nerve) and no sex differences were apparent. These growth-cone-like profiles were often continuous with a vesiculated region of the axon (Fig. 2). Profiles resembling lamellipodia were not seen in nerves from tadpole stage 66 or in juvenile or adult nerves.

Myelination

We wished to compare the overall maturation of the laryngeal nerve in males and females by examining the progressive myelination of axons. Some examples of myelinating and myelinated axons are shown in Figure 2. At stage 52, all axons are unmyelinated (Table 1). The nerve has numerous large axons ($2-9 \mu^2$) separated by smaller axons ($<1 \mu^2$). Five axons (two large, three small) are surrounded by glial processes. The first axons to become completely myelinated in the laryngeal nerve occur by stage 54. These axons are large in diameter ($4-6 \mu^2$) and are located at the nerve periphery. At this stage, the nerve also contains large and small unmyelinated axons.

The subsequent time course of myelination is given in Table 1. The number of myelinating axons is low (2–6%) at stage 56 but then increases rapidly at stages 62 (15%) and 66 (33%). Completely myelinated axons are infrequent at early stages (56: 2–3%; 59: 3–4%; 62: 4–5%) but increase sharply in frequency by stage 66 (22–30%). By the juvenile stage, all axons are completely myelinated. The process of myelination is virtually identical for males and females. We should note that at juvenile and adult stages, the laryngeal nerve also contains 10–20 fascicles of very small ($<1 \mu^2$) unmyelinated axons, 1–15 axons per fascicle. One nerve of a metamorphic male also contained 8 such fascicles. These are most probably sensory processes innervating the tendon of the laryngeal muscle or the glottis (the larynx does not contain any muscle spindles).

Necrosis

Axon profiles containing dark inclusions, dilated and electron-dense mitochondria, or dense lamellar structures were found in nerves of tadpoles; no

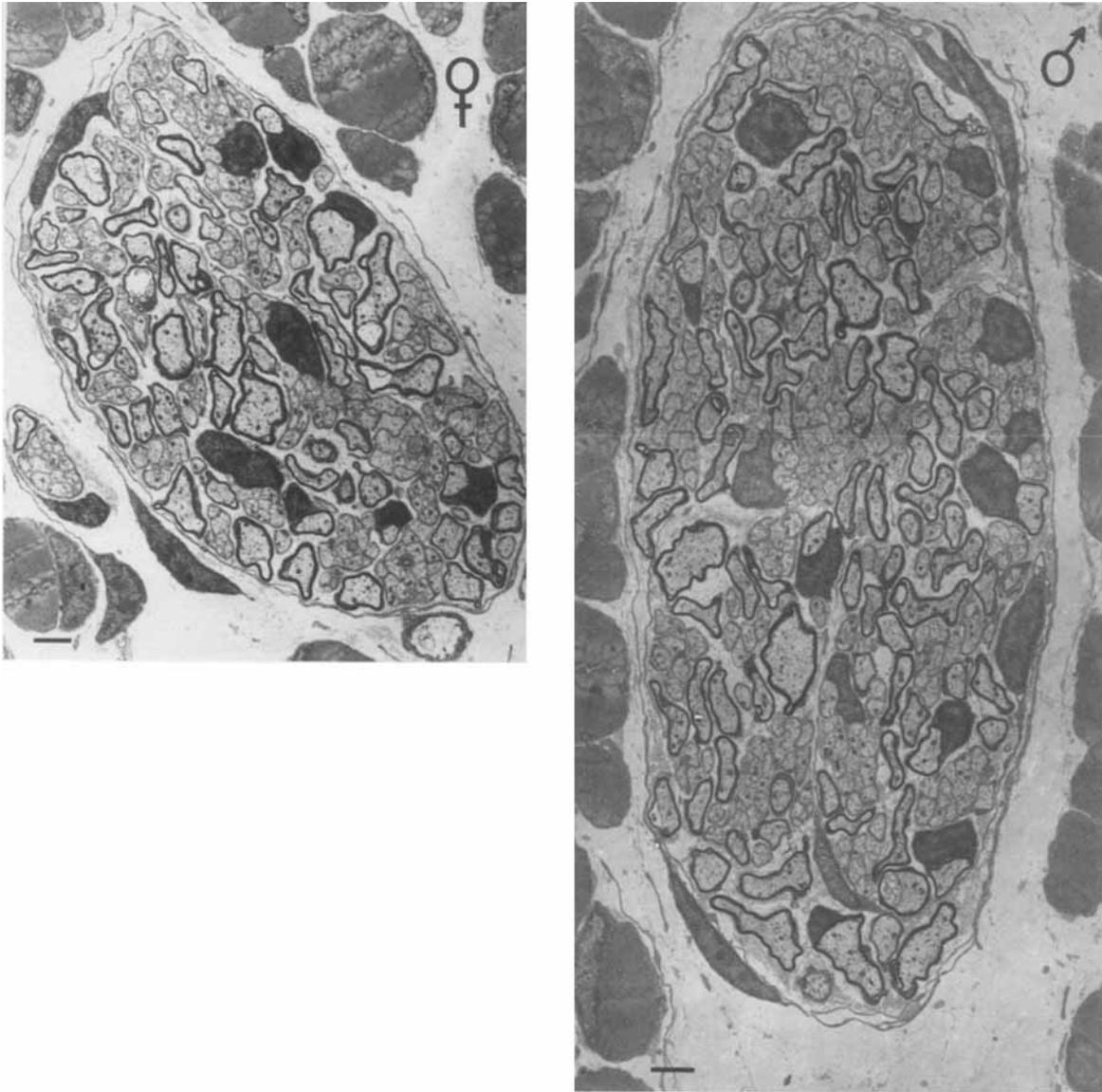


Figure 4 Electron micrographs of the laryngeal nerve in female (left) and male (right) *X. laevis* at metamorphosis (tadpole stage 66). The gross dimorphism in size of the nerve is due to the larger number and size of axons in the male. Scale bars = 2 μ .

evidence of axonal degeneration was seen after metamorphosis. Some examples of necrotic axons in laryngeal nerves of the two females at tadpole stage 62 are shown in Figure 3. These axons are the most clearly necrotic seen in any laryngeal nerve. The axoplasm is disrupted and electron-dense, dark inclusions (probably mitochondria) are present; the disrupted axons are entirely surrounded by a glial process. We also observed many examples of electron-dense mitochondria within axons that appeared to be intact (Fig. 3). Because such mitochondria are said to represent the earliest

stages of axonal degeneration (Williams et al., 1986), we counted such axons at every stage of development in both sexes (values were normalized as percentages of axonal profiles containing at least one mitochondrion). No consistent differences by age or sex were apparent.

SUMMARY

We examined electron micrographs of developing laryngeal nerves for qualitative evidence of sex

differences in axonal maturity (possibly reflecting the arrival of axons at the larynx) and of axonal degeneration (possibly reflecting the process of axon loss). The hypothesis that new axons arrive at the larynx between tadpole stages 56 and 62 is supported by the presence of growth cone-like axonal profiles and the continued presence of large numbers of small, probably immature, axons. No definitive support for the extra axon outgrowth thought to occur in males between stages 59 and 62 was found. Signs of axonal degeneration were present throughout tadpole stages in both sexes but were rare; it is possible that they reflect death of the parent cell body. Signs of axon degeneration coexist with increases in axon numbers suggesting that the two processes are concurrent.

DISCUSSION

Developing motor neurons are characterized by an overproduction of cells followed by a wave of cell death after contact with developing muscle targets is established (Oppenheim, 1985). Because young

motor neurons usually send only one axon to the periphery, the processes of axon outgrowth and death of motor neurons are reflected in the number of motor axons in a specific motor nerve (Prestige and Wilson, 1972). The overall pattern of axon number in the developing laryngeal nerve of *Xenopus laevis* conforms to this classical pattern. Counts at tadpole stages 52 and 54 suggest that axon numbers are rising due to initial outgrowth. Axon counts then peak and decline reaching stable adult values between metamorphosis and the juvenile stage; the decline in axon number fits the pattern associated with ontogenetic cell death of parent motor neurons.

When data for males and females are examined separately, however, it is clear that axon numbers in developing laryngeal nerve of male *X. laevis* diverge from the usual pattern. At tadpole stage 56, when the sexes can first be distinguished, axon numbers are the same for males and females. Axon number then increases in males between stages 59 and 62. Before stage 59 and after stage 62, male axon numbers are decreasing; by the juvenile stage, adult values are attained. In females, axon

Table 1 Morphological Characteristics of Developing Laryngeal Nerves in Male and Female *Xenopus Laevis*

Stage	Sex	Mean Total Number of Axons	Mean Number of Unmyelinated Axons ²	Mean (Range) Number of Myelinating Axons ²	Mean (Range) Number of Myelinated Axons ²
52 ¹	?	277	272	5	0
54 ¹	?	552	545	3	4
56	M	611	568	28.8 (4-46)	14 (10-20)
	F	617	589	10.3 (4-18)	17.5 (14-23)
59	M	563	522	21.5 (5-44)	19.3 (18-22)
	F	467	424	25.8 (19-43)	17.3 (8-27)
62	M	681	554	88 (43-155)	28.3 (19-33)
	F	480	435	71.8 (60-91)	26.8 (22-31)
66	M	455	171	147 (115-182)	137.3 (89-220)
	F	400	172	138 (74-205)	89.8 (45-137)
PM	M	301	0	0	301
5	F	241	0	0	241
PM	M	321	0	0	321
6	F	225	0	0	225

¹ Tadpoles at these stages (one each) could not be sexed as gonads are not yet differentiated.

² The number of axons evincing the appropriate morphological characteristics was counted on electron micrographs through the laryngeal nerve for males and females at each stage.

number drops sharply between stages 56 and 59, plateaus between 59 and 62 and then continues to decrease (in parallel with male values) until a stable number is reached at the juvenile stage.

These findings draw attention to three issues. What cellular events are responsible for axon addition and loss? Why are addition and loss different for the sexes? What are the consequences of sex differences in tadpole axon numbers for the later sexual differentiation of laryngeal muscle?

Mechanisms of Axon Addition and Loss

Two cellular processes account for changes in axon numbers: axon loss and axon addition. Axon loss is inferred from decreases in axon numbers and the occurrence of necrotic axons. Axon addition is inferred from increases in axon numbers and from the presence of growth cones and of large numbers of small axons. Our ultrastructural evidence suggests that axon loss and addition are concurrent in the tadpole laryngeal nerve. What are the relative contributions of these processes to sex differences in the laryngeal nerve? The question is of some importance because either process, if sufficiently exaggerated, could account for sexually dimorphic axon numbers.

We attempted to determine the relative contributions of loss and addition to sex differences in axon numbers by examining indices of axonal maturity (growth cones, axon size, myelination) and death (degeneration). Axon loss could also be attributable to retraction, an event for which we know of no reliable ultrastructural correlate. Analysis of growth cones was limited by the very small number present in a single section; no sex differences were apparent. Myelination was robust and easily quantified; no sex differences were seen. Axon sizes were similar in males and females until metamorphosis. We saw very few frankly degenerating axons (except in female tadpoles at stage 62). We thus cannot unequivocally assign sex differences in number at a given stage to particular contributions of loss and addition. Because of concurrent axon loss, estimates of axon addition are likely to be underestimates. Similarly, the arrival of additional axons from the brain may mask an underlying process of axon loss.

At stage 56, males and females have the same number of axons in the laryngeal nerve. We have shown (Gorlick and Kelley, 1987) that neurogenesis of laryngeal motor neurons is complete by stage 56 and is the same for males and females. Together, these data suggest that males and fe-

males have the same number of laryngeal motor neurons at tadpole stage 56. Could the pattern of axon numbers observed here be attributable solely to sex differences in axon loss due to the death of the parent motor neuron? Overall loss of axons is greater in females than in males suggesting that females may experience more cell death. On the other hand, males do show a significant increase in axon numbers between tadpole stages 56 and 62. This gain cannot be the result of a momentary pause in axon loss, which would halt, but not reverse, declining axon numbers. Instead, additional axons must arrive from the central nervous system (CNS) during this period. Thus although we cannot rule out sex differences in ontogenetic cell death as the sole factor controlling axon numbers, our evidence suggests that axon addition also plays a role in establishing sex differences in axon number.

What is the source of the additional axons that arrive at the male larynx between stages 56 and 62? One possibility is that additional laryngeal motor neurons are produced in males and that their axons arrive at the larynx during this time. We have examined neurogenesis in the laryngeal motor nucleus of *Xenopus laevis* (Gorlick and Kelley, 1987) but our data do not support this hypothesis. Developing frog embryos received injections of tritiated thymidine from gastrulation through the onset of metamorphosis and were examined at tadpole stage 64 when the laryngeal motor nucleus is recognizable. At no stage during development did we see evidence for the production of more laryngeal motor neurons in males than in females.

Another possibility is that males and females have the same number of laryngeal motor neurons but that in males these neurons sprout additional axon collaterals that arrive at the larynx between stages 59 and 62. Axon collaterals have been described in developing motor and autonomic nerves of chicks and ducklings (Landmesser and Pilar, 1976; Chu-Wang and Oppenheim, 1978, Sohal and Weidman, 1978). These collaterals (which can be seen when the nerve is sectioned longitudinally) are associated with vesiculated axon profiles. Could such collaterals account for sex differences in laryngeal axon numbers in *Xenopus laevis* tadpoles? We sectioned tadpole laryngeal nerves just before entry into muscle and observed small numbers of vesiculated axon profiles, similar in appearance to profiles seen in developing chick and duck nerves in both males and females; these vesiculated axon profiles may repre-

sent collateral axons of laryngeal motor neurons. Because vesiculation is associated with the collateral branch point only, the number of vesiculated axonal profiles in a single nerve section is an underestimate of the total number of collaterals especially if, as in duck trochlear nerve, axon collaterals are short and do not reach muscle targets (Sohal and Weidman, 1978). We thus cannot estimate the overall prevalence of axon collaterals in the laryngeal nerve. To determine if there was a sex difference in number of axon collaterals, we counted the number of vesiculated axon profiles in males and females at each tadpole stage. Because we did not observe any consistent sex differences in vesiculated axons of any stage—either in absolute number or in percentage of total axons—we do not favor the hypothesis that the greater number of axons in male laryngeal nerves is due to increased collateral sprouting.

A final possibility is that preexisting neurons are induced in males to direct or redirect axonal processes to laryngeal muscle. A similar process (though without a sex difference) has been suggested for the lumbar motor neurons of bullfrogs (Farel, 1987). In juvenile *Rana catesbeiana*, the lumbar motor column contains mature motor neurons and cytologically less differentiated cells that resemble a type of motor neuron found in tadpoles. As the frog grows, the numbers of these less differentiated neurons (which do not send axons to the leg) decreases and the number of mature motor neurons increases. These less differentiated cells could represent a reserve of postmitotic potential motor neurons. The neurons may be synaptically immature and lack an axon or they may have established axonal arbors that retract and are redirected towards peripheral muscle targets. In the case of laryngeal motor neurons in *Xenopus laevis* we would also have to postulate that the signal evoking axonal outgrowth to the muscle is more compelling in males than in females.

In developing *X. laevis*, motor neuron loss occurs at similar stages for different CNS nuclei despite the differential development of their target muscles (Prestige and Wilson, 1972; Schonberger, Escher, and Van der Loos, 1986). This coincidence suggests the participation of a systemic factor; pharmacological blockade of the hormone thyroxine has been shown to prevent axon loss in the oculomotor nerve, which resumes once the blockade is lifted (Schonberger and Escher, 1988). The *X. laevis* laryngeal nerve shows a pattern of axonal loss similar to oculomotor and spinal

nerves: the decline in axon numbers begins between stages 56 and 59 and is steepest between 62 and 66 when maximum titers of thyroxine occur (Dodd and Dodd, 1976; Leloup and Buscaglia, 1977). The timing of axon loss is the same for males and females. We thus suggest that laryngeal axon loss is initiated and maintained by secretion of thyroxine, which is the same for males and females. Note that, although the timing of laryngeal axon loss is the same for the sexes, the extent of axon loss is not. Between tadpole stages 56 and adulthood, net axon number decreases in males by 47% and in females by 64%. Myelination of the laryngeal nerve is also probably controlled by thyroxine as it is for the oculomotor nerve (Schonberger and Escher, 1988). Again, we suggest that myelination (identical for male and female laryngeal nerves) is controlled by thyroxine secretion which is the same for the sexes.

Why is Axon Outgrowth Greater and Axon Loss Less in Males than in Females?

The signal evoking axonal outgrowth must be effective before stage 62 and be more compelling in males than in females. This signal could be steroid secretion from the newly differentiated gonads. The major gonadal androgens of male *X. laevis* are testosterone and dihydrotestosterone (Kelley, 1980; Wetzel and Kelley, 1983). Morphological evidence suggests that the gonad is competent to synthesize testosterone-precursor androgens starting at stage 57 (Witschi, 1971). The greater number of axons in male *X. laevis* could thus be due to more androgen secretion by the newly differentiated male gonads. Androgen may act directly on target cells in the laryngeal motor nucleus to induce axonal outgrowth along the pathways established by existing laryngeal motor neurons. Androgen binding is detectable in laryngeal motor neurons at tadpole stage 64 but not at tadpole stage 60 (Gorlick and Kelley, 1986), suggesting that direct sensitivity of these neurons to androgen arises at this time.

What accounts for the differential rates of axon loss in males and females? These differential rates do not appear until after stage 56 at which time male and female gonads are cytologically differentiated. Again, we suggest that the lesser degree of axonal loss in males is due to greater secretion of androgen after stage 56. We presume that androgen increases the amount or availability of some molecule or type of cell interaction otherwise in

such short supply as to limit survival of motor neurons.

Relation to Other Sexually Dimorphic Neuromuscular Systems

The ontogeny of the laryngeal neuromuscular system in *Xenopus laevis* can be compared to the development of the sexually dimorphic motor neurons (perineal or SNB neurons) that innervate the levator ani and bulbocavernosus muscles in rats. Neurons of the SNB are present in both sexes at birth but are lost in females unless they are supplied with exogenous androgen during the perinatal period (Breedlove and Arnold, 1981). Androgen acts to reduce the normal ontogenetic cell death that male SNB neurons would otherwise experience and also acts to encourage the migration of these cells into the SNB motor nucleus (Nordeen et al., 1985; Sengelaub and Arnold, 1986). Prevention of ontogenetic cell death is believed to result primarily from preservation of the target muscles of the SNB neurons by androgen (Fishman and Breedlove, 1988; Fishman et al., 1990).

Our data on the developing laryngeal nerve of *Xenopus laevis* suggest that axon loss is less in males than in females. We do not yet know whether axon loss is due to death of the parent motor neuron. Sex differences in ontogenetic cell death cannot, however, entirely account for the origins of sex difference in innervation of the *X. laevis* larynx. Some new axons are extended by neurons in the male brain. Comparable studies of axon numbers in the rat SNB system have not been carried out and we thus cannot exclude the possibility that hormone-evoked axonal outgrowth occurs in other sexually dimorphic neuromuscular systems.

Axon Addition and the Sexual Differentiation of Laryngeal Muscle

We can summarize our working hypothesis as follows. When the gonads of *X. laevis* differentiate at tadpole stage 56, steroids are secreted; androgen secretion is greater in males than in females. The greater amounts of circulating hormone in males are responsible for maintaining existing axons and for inducing additional axon outgrowth. Do the additional axons in the laryngeal nerve play any role in the subsequent sexual differentiation of the larynx?

One possibility is that additional male axons contribute to the male-specific pattern of laryngeal

muscle fiber addition. Between metamorphosis and 6 months postmetamorphosis, males add about 150 new laryngeal muscle fibers per day (Marin et al., 1990). Females add only about 25 fibers per day. The new muscle fibers may derive from a population of androgen-sensitive, secondary myoblasts (Sassoon and Kelley, 1986; Kelley et al., 1989). In chick and rat, some late or secondary myoblasts require innervation for proliferation (McLennan, 1983; Ross, Duxon, and Harris, 1987). Thus it is possible that the extra axons in male laryngeal nerve contribute to the production of androgen-sensitive myoblasts and thus to the determination of the adult male number of muscle fibers.

In summary, we have shown that adult sex differences in numbers of laryngeal motor axons result from a dimorphic developmental program in which males both produce more axons and have more axons that persist until adulthood. The timing of axon loss and the development of myelination is not sexually dimorphic and could be controlled by thyroxine secretion. The degree of axon loss and the arrival of new axons are sexually dimorphic, perhaps due to greater secretion of androgenic steroids from the newly developed male gonad. Androgen could maintain axons by acting either on the motor neuron or on the muscle target. Androgen may act to induce axon outgrowth by stimulation of collateral sprouting or by alteration of the axonal trajectory of cells that would otherwise have remained immature or have formed synapses elsewhere. We speculate that the presence of additional axons between stages 59 and 62 in males is required for the survival and proliferation of the androgen-sensitive myoblasts that produce more male muscle fibers in post-metamorphic development.

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