

# Trophic Effects of Androgen: Development and Hormonal Regulation of Neuron Number in a Sexually Dimorphic Vocal Motor Nucleus

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**ABSTRACT:** In *Xenopus laevis*, the laryngeal motor nucleus (n. of cranial nerves IX-X) is part of a sexually differentiated, androgen sensitive neuromuscular system devoted to vocalization. Adult males have more n. IX-X neurons than females; however, during development of n. IX-X, the rate of neurogenesis does not appear to differ between the sexes. In this study, we explored the role of naturally occurring cell death in the development of this nucleus and asked whether cell death might be involved in establishing the sex difference in neuron number. Counts of n. IX-X neurons reveal that at tadpole stage 56, males and females have similar numbers of n. IX-X neurons, but by stage 64 male neuron numbers are greater. This sex difference arises owing to a greater net loss of neurons in females—males lose ~25% of their n. IX-X neurons between stages 56 and 64, while females lose ~47%. Sexual differentiation of n. IX-X neuron number coincides with a period of developmental cell death, as evidenced by terminal transferase-mediated dUTP nick-end labeling and the presence of pyknotic nuclei in n. IX-X. A role for

gonadal hormones in controlling cell number was examined by treating tadpoles with exogenous androgen and determining the number of n. IX-X neurons at stage 64. Dihydrotestosterone (DHT) treatment from the beginning of the cell death period (stage 54) until stage 64 had no effect on the number of n. IX-X neurons in males but did significantly increase n. IX-X neuron number in females. This increase was sufficient to abolish the sex difference normally observed at stage 64. Although DHT induced increases in female neuron number, it did not induce increases in cell proliferation or addition of newly born neurons to n. IX-X. DHT may therefore have increased neuron number by protecting cells from death. We conclude that androgens can influence the survival of n. IX-X neurons during a period of naturally occurring cell death, and that this action of androgen is critical to the development of sex differences in n. IX-X neuron number. © 1999 John Wiley & Sons, Inc. *J Neurobiol* 40: 375–385, 1999

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Gonadal steroid hormones have been implicated in the sexual differentiation of certain behaviors and of the anatomical structures that subserve these behaviors (Kelley, 1988; Breedlove, 1992). For example, in the African clawed frog *Xenopus laevis*, courtship behavior involves sexually dimorphic vocalizations—only adult males produce the advertisement call, a song

that attracts and excites sexually receptive females (reviewed in Kelley and Tobias, 1999). Expression of this behavior by adults requires exposure to androgens, both at the time of calling and during development (Wetzel and Kelley, 1983; Watson et al., 1993; Kelley, 1996).

The anatomical substrate for vocal behavior in *Xenopus* includes a neuromuscular system composed of the larynx (the vocal organ) and motor neurons located in the brain-stem nucleus of cranial nerve IX-X (n. IX-X). Calls are produced by contraction of

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laryngeal muscles in response to activity in the n. IX-X motor neurons that innervate the larynx (Kelley and Tobias, 1999). Both central and peripheral components of this neuromuscular system have been shown to be sexually dimorphic in adult frogs. In the periphery, laryngeal muscle differs dramatically between adult males and females, as does the laryngeal synapse (Sassoon and Kelley, 1986; Sassoon et al., 1987; Tobias et al., 1995). In the central nervous system (CNS), male n. IX-X cells are larger in somal size and dendritic extent; males also have more n. IX-X neurons and more axons innervating the larynx than females (Kelley et al., 1988; P. Hannigan and D. B. Kelley, unpublished; Kelley and Dennison, 1990).

Masculinization of the vocal neuromuscular system, and the behaviors that it controls, require gonadal androgen secretion during development (reviewed in Kelley, 1996). In laryngeal muscle, the timing and hormonal regulation of sexual differentiation have been characterized (Sassoon and Kelley, 1986; Sassoon et al., 1987; Tobias and Kelley, 1995). Much less is known, however, about the development of sex differences in the CNS component of this neuromuscular system. In this study, we examine the development and hormonal control of sex differences in the number of neurons present in n. IX-X, the motor nucleus that contains the laryngeal motor neurons.

A major mechanism by which gonadal hormones establish sex differences in CNS structure and function during development is through regulation of neuron number (Arnold, 1992; Breedlove, 1992; Kelley, 1988). Hormones have been shown to influence neuron number both by controlling incorporation of newly born neurons into sexually dimorphic CNS regions and by manipulating the rate of cell death in these regions (Kirm and DeVoogd, 1989; Burek et al., 1997; Konishi and Akutagawa, 1987; Nordeen et al., 1985; Davis et al., 1996; Arai et al., 1996). A survey of neurogenesis in the *Xenopus* vocal pathway revealed that n. IX-X neurons are born between tadpole stages 11 and 62; sex differences in the number of neurons generated were not apparent (Gorlick and Kelley, 1987). It is thus unlikely that n. IX-X neuron number becomes sexually differentiated as the result of more neurons being generated in males. On the other hand, several lines of evidence suggest a link between androgens, cell death, and sexual differentiation of n. IX-X neuron number. In juvenile frogs, androgen treatment has been shown to rescue n. IX-X neurons from axotomy-induced cell death, indicating that androgens are capable of intervening in the cell death program (Perez and Kelley, 1996). Evidence

that androgens might influence cell death during development comes from studies of the number of motor axons innervating the larynx. Between late tadpole stages and adulthood, the number of laryngeal axons declines more in females than in males (Kelley and Dennison, 1990), suggesting a possible sex difference in survival of the motor neurons that give rise to these axons. Treatment with exogenous androgen during this period increases laryngeal axon number in both sexes (Robertson et al., 1994). This result could indicate a survival-promoting effect of androgen on the motor neurons that project to the larynx, or it could simply reflect an androgen-induced increase in axon outgrowth and collateral sprouting.

In this study, we sought to clarify both the normal pattern of developmental changes in cell number and the ability of androgens to affect this pattern, by directly counting cell bodies in n. IX-X rather than relying on counts of axons in the laryngeal nerve. Our counts reveal that immediately following sexual differentiation of the gonad, n. IX-X neuron number does not differ between the sexes; thereafter, neuron number declines substantially in both sexes, with a greater loss observed in females. *In situ* DNA fragmentation assays [terminal transferase-mediated dUTP nick-end labeling (TUNEL) histochemistry] suggest that the cell loss we observed is due to naturally occurring cell death. Treatment with exogenous androgen during the cell death period reduces the expected loss of n. IX-X cells in females, thereby preventing the initial establishment of sex differences in neuron number. Together, our results indicate that n. IX-X neuron number is sexually differentiated by androgen-mediated rescue from cell death.

## MATERIALS AND METHODS

### Animals and Histology

*Xenopus laevis* tadpoles were obtained from Nasco (Ft. Atkinson, WI) and were staged according to the criteria of Nieuwkoop and Faber (1956). They were deeply anesthetized by immersion in 0.1% MS-222 (Sigma) and sacrificed by perfusion with 0.1 M phosphate buffer, followed by phosphate-buffered 4% paraformaldehyde. Brain, spinal cord, and interrenal glands with attached gonads were collected from each animal. In older animals (stages 64 and 66), the CNS was dissected free of the skull and vertebral column, but in younger animals (stages 54 and 56) the entire head and spinal region were collected for histological processing. CNS and gonads were postfixed in 4% buffered paraformaldehyde for 5–8 h, washed overnight in 0.1 M PO<sub>4</sub> buffer, and then embedded in paraffin following dehydration in ethanol and clearing in methyl salicylate. Hori-

zontal sections through the CNS were cut at 10  $\mu\text{m}$  and were either stained with Cresyl violet or processed for TUNEL histochemistry. Ten-micrometer transverse sections through the interrenal–gonad complex were stained with hematoxylin and eosin and were used to determine the sex of each tadpole.

Adult frogs were anesthetized by MS-222 injection and perfused as above. Brains were removed and postfixed overnight in buffered 4% paraformaldehyde, after which they were washed, embedded in paraffin, and sectioned. Sex was determined by external characteristics and by examination of the gonads upon sacrifice.

## Androgen Treatment

To allow comparison of our results with those obtained by Robertson et al. (1994), we adopted the same regimen of androgen treatment. Dihydrotestosterone (DHT; Sigma) was dissolved in 95% ethanol and added to tank water to give a final DHT concentration of 1  $\mu\text{g/L}$ . Starting at stage 54, tadpoles received daily changes of tank water containing either DHT or ethanol vehicle. The final ethanol concentration was 0.0095%. Treatment continued until tadpoles reached stage 64 (about 30 days), at which time they were sacrificed and processed as described above. For all four treatment groups,  $n = 4$ : DHT–male, DHT–female, vehicle–male, vehicle–female.

In another experiment, tadpoles ( $n = 5$  of each sex) received DHT or vehicle treatment as described above, except that after 8 days of treatment, they received injections of 10  $\mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine (NEN) (Cohen and Kelley, 1996). Tadpoles were then reared to stage 64 in untreated tank water and sacrificed without perfusion. Tissue was fixed in 10% formalin for 2–3 days, embedded in paraffin, and sectioned as described. Following dewaxing and rehydration, slides were dipped in NTB2 autoradiographic emulsion (Kodak), exposed for 7 weeks at 4°C, then developed using Kodak D-19 developer and counterstained with Cresyl violet.

## TUNEL Histochemistry

Stage 54 tadpoles ( $n = 6$ ), stage 56 tadpoles ( $n = 4$  of each sex), stage 64 tadpoles ( $n = 4$  each sex), 1 week postmetamorphic frogs ( $n = 4$  each sex), and adults ( $n = 1$  female) were processed as described above and analyzed by TUNEL histochemistry.

Slides were dewaxed in xylene and rehydrated. Tissue was permeabilized by microwave-irradiating slides for 5 min on low power in a 200-mL bath of citrate buffer (1  $M$  NaCl, 0.1  $M$  Na citrate, pH 6.4), followed by 25 min treatment with Proteinase K (40  $\mu\text{g/mL}$ ) (Boehringer Mannheim) at room temperature. After thorough washing with Tris-buffered saline (TBS) (25  $mM$  Tris, 140  $mM$  NaCl, 3  $mM$  KCl, pH 7.4), sections were incubated for 75 min at 37°C with a TUNEL labeling mix containing terminal deoxynucleotidyl transferase (TdT) and fluorescein-conjugated nucleotides (Cell Death Detection Kit; Boehringer

Mannheim). The signal was visualized using an alkaline phosphatase (AP)–conjugated anti-fluorescein antibody (Boehringer; incubation 40 min at 37°C), followed by an AP-mediated substrate reaction (HistoMark Red; Kirkegaard Perry Laboratories).

## Cell Counts

We chose to examine n. IX-X number at three developmental time points in addition to adulthood. Stage 54 marks the beginning of thyroxine secretion, an event that is required in *Xenopus* for (a) initiation of metamorphosis, (b) initiation of motor neuron death in the spinal cord, and (c) initiation of axon loss from the laryngeal nerve (Leloup and Buscaglia, 1977; Prestige and Wilson, 1972; Robertson and Kelley, 1996). Stage 56 is the first stage at which the gonads have differentiated sufficiently to allow determination of sex, and it is also the last age at which the number of axons innervating the larynx is sexually monomorphic. Stage 64 represents a late time point in the metamorphic climax, and it immediately follows the end of n. IX-X neurogenesis (Gorlick and Kelley, 1987). The number of n. IX-X neurons was determined from Cresyl violet–stained 10- $\mu\text{m}$  sections. Slides were coded to obscure the sex and treatment group of each animal. In most animals n. IX-X was counted unilaterally; bilateral counts of selected animals revealed no systematic differences in cell number between the hemispheres. The total number of cells reported is the unilateral count multiplied by two to account for the other side of the medulla.

We used conservative cell counting criteria designed to exclude nonneuronal cells and prevent counting the same cell twice. To this end, the following requirements for counting a cell were established: (a) Only cells with a visible nucleus (stages 54 and 56) or nucleolus (stage 64 and adult; nucleoli were harder to detect in smaller, less differentiated cells) were counted. (b) Because the typical neuron morphology includes a large cell body, all cells were required to have stained cytoplasm visible in addition to the nucleus. This criterion excluded small cells, including glia, from our counts. (c) Only cells that were obviously positioned within n. IX-X were counted. At all ages included in this study, n. IX-X is easily recognized by its position in the white matter lateral to the reticular formation, just caudal to the point where cranial nerve IX-X enters the skull (Simpson et al., 1986) (Fig. 1). Only cells that were clearly positioned in the white matter, with distinct separation from the reticular formation, were counted. Cells rostral to the entry point of the fourth root of nerve IX-X were excluded. Our morphological criteria for defining n. IX-X neurons were confirmed by injections of horseradish peroxidase (HRP) into the laryngeal muscle of tadpoles, which resulted in retrograde labeling of neurons in the region we have identified as n. IX-X. HRP labeling was seen only in cells that fit all of our criteria for inclusion in the counting data (not shown).

Neuron counts were performed without the use of stereological methods, and therefore our counts may be subject

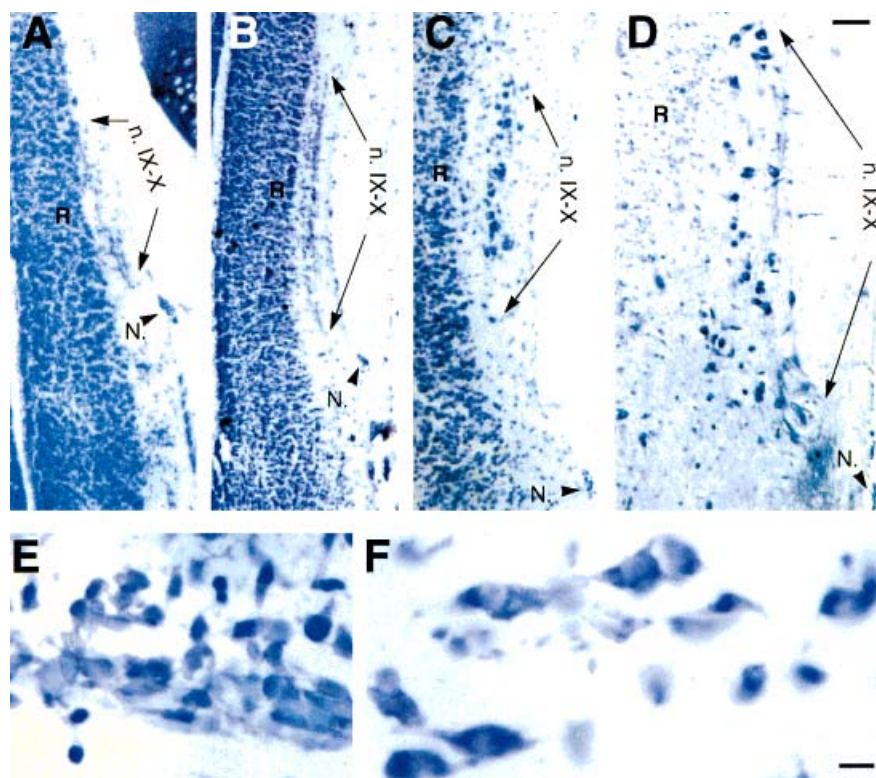


Figure 1

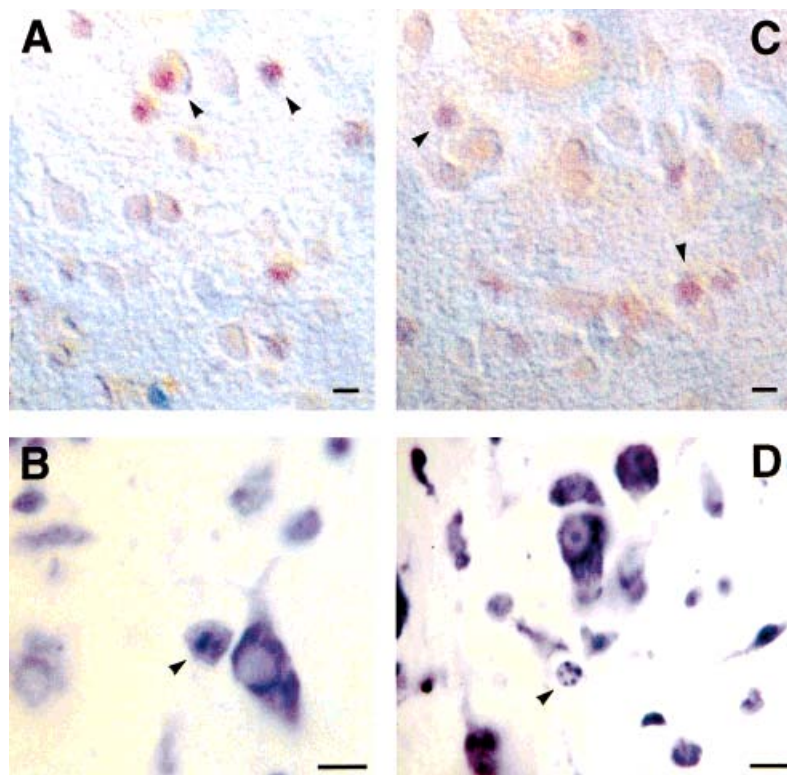


Figure 3

to bias introduced by sectioning or counting methodology (West, 1993). However, we have taken precautions to reduce the possibility that our conclusions could be compromised by such bias. For example, bias may be introduced if the objects being counted are larger than the section width; in this case, a single object may appear in more than one section and be counted as two objects (West, 1993). To avoid this problem, we chose to count only every other section. The largest cell nuclei in n. IX-X neurons were  $\sim 10 \mu\text{m}$  in their longest dimension [see Figs. 1(F) and 3(B,D) for representative neurons]. We sampled every second  $10\text{-}\mu\text{m}$ -thick section, thereby ensuring that the odds of counting the same cell twice were very small.

This methodology, while effective for preventing overcounting, may lead to underestimates of cell number owing to the presence of cells in skipped sections. As long as the size of the counted object (in this case, cell nuclei) does not vary across sample groups, this underestimate should apply with equal probability to all groups, and thus should not bias the data. We measured the nuclear diameter of n. IX-X neurons and found that nuclear size does not vary with sex in tadpoles of the same age. The mean nuclear diameter at stage 56 was  $5.45 \pm 0.26 \mu\text{m}$  in males and  $5.67 \pm 0.22 \mu\text{m}$  in females; at stage 64, the mean diameter was  $6.36 \pm 0.17 \mu\text{m}$  in males and  $6.15 \pm 0.15 \mu\text{m}$  in females [mean  $\pm$  standard error of the mean (S.E.M.);  $n = 30$  cells chosen randomly from three separate animals]. These differences were not significant by a one-tailed  $t$  test ( $p > .1$  for both stage 56 and 64). Thus, nuclear size in n. IX-X is equivalent in male and female tadpoles of the same age; we therefore find that our data are suitable to allow comparison of cell number between these groups. A similar analysis revealed minimal effects of DHT treatment on nuclear size: In males, treatment had no effect (vehicle-treated,  $6.36 \pm 0.17 \mu\text{m}$ ; DHT-treated,  $6.19 \pm 0.16 \mu\text{m}$ ), while in females, treatment actually produced a small but statistically significant decrease in nuclear diameter (vehicle-treated,  $6.15 \pm 0.15 \mu\text{m}$ ; DHT-treated,  $5.73 \pm 0.12 \mu\text{m}$ ).

Cell size does vary with age, however, and adult males

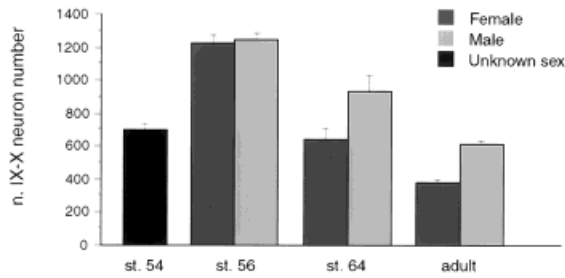
are known to have a larger mean n. IX-X cell size than females (Kelley et al., 1988). Because smaller objects have a higher probability of being missed in skipped sections, differences in cell size are a potential source of bias when comparing these groups. However, any such bias is unlikely to have affected our conclusions, for two reasons. First, we found that cell number decreased between stage 56 and adulthood, despite increases in cell (and nuclear) size over that time. This result demonstrates that our counting methodology was not unduly sensitive to differences in size, and that any bias introduced by such differences did not compromise our conclusions when comparing across age groups. The second reason we do not consider our counting data biased is that our cell count data correspond well to previous counts of axons in the laryngeal nerve over development, in adulthood, and following DHT treatment (Kelley and Dennison, 1990; Robertson et al., 1994). Such axon counts are not biased, since in a cross-section of nerve, each axon gives rise to exactly one sectional profile. The sex differences in adult neuron number we observed are corroborated by sex differences in adult axon number observed by Kelley and Dennison (1990). Also, changes in axon number over development and following DHT treatment are similar to changes in neuron number we observed (see Results). The fact that our data are consistent with axon count data suggests that the cell counting methodology we used was appropriate to allow comparisons across age, sex, and treatment group.

## Statistical Analysis

For each sex and treatment, four animals were used for n. IX-X neuron counts, with the following exceptions: adults ( $n = 12$  for each sex); stage 56 tadpoles ( $n = 4$  males;  $n = 3$  females), and stage 54 tadpoles ( $n = 5$  of unknown sex). For TUNEL cell counts,  $n = 4$  of each sex at each stage (56 and 64). The significance of differences in cell count data were assessed by the two-tailed Mann-Whitney

**Figure 1** n. IX-X at tadpole stage 54 (A), stage 56 (B,E), stage 64 (C,F) and adulthood (D). (A–D) Low-power view showing the location of n. IX-X in Nissl-stained sections. Caudal is up, rostral is down, medial is to the left, and lateral to the right in each panel; all panels are equally magnified. At each age, the nucleus is readily identifiable by its location within the white matter lateral to the reticular formation and caudal to the point at which nerve IX-X (N.) enters the brain. The nucleus takes on a more adultlike morphology between stages 56 and 64; notably, it becomes relatively shorter in the rostrocaudal dimension and cell density decreases. These changes suggest a possible decrease in cell number with age. (E,F) Higher-power view of n. IX-X neurons at stage 56 (E) and stage 64 (F). Rostral is to the left, lateral is down, and magnification is equal in each panel. At stage 56 (E), cells are tightly packed, but at stage 64 (F), cell density is greatly decreased. N. = nerve IX-X; R = reticular formation. Scale bar = 0.5 mm (A–D); 0.1 mm (E,F).

**Figure 3** Cell death in n. IX-X neurons at stage 64. (A,C) Cell death assessed by TUNEL staining in representative sections through male (A) and female (C) n. IX-X; photographs were taken using Nomarski optics. Red-colored nuclei indicate the presence of fragmented DNA. Some examples of labeled cells are marked by arrowheads. (B,D) Nissl-stained sections from male (B) and female (D) stage 64 tadpoles showing the presence of pyknotic nuclei (arrowheads). Scale bars = 0.1 mm.



**Figure 2** Ontogeny of neuron number in n. IX-X. At stage 56, neuron number is at its peak in both males and females; there is no significant sex difference ( $p = .86$ ). At stage 64 and in adulthood, males have significantly more neurons than females (stage 64:  $p < .05$ ; adult:  $p < .0001$ ). Between stages 56 and 64, and again between stage 64 and adulthood, neuron number declines significantly in both sexes (56 vs. 64 male:  $p < .025$ ; 56 vs. 64 female:  $p < .05$ ; 64 vs. adult male:  $p < .01$ ; 64 vs. adult female:  $p < .01$ ). At stage 54, tadpole sex cannot be determined; n. IX-X neuron number is significantly lower at this stage than at stage 56 ( $p < .02$ ).

*U* test (Statview; Abacus Concepts).  $p$  values  $< .05$  were considered as significant.

## RESULTS

### Ontogeny of Sex Differences in n. IX-X Neuron Number

In this experiment, we asked whether the number of neurons in n. IX-X fluctuates during development, and whether these fluctuations differ between the sexes. Our cell counts revealed that a substantial decline in neuron number occurred in both males and females (Fig. 2). After peaking at stage 56, the number of n. IX-X neurons dropped sharply during the ~3 weeks between stages 56 and 64 ( $p < .0025$  for males;  $p < .05$  for females). The decline in cell number continued during the ~24 months between stage 64 and adulthood ( $p < .01$  for both sexes).

Although both sexes exhibited a significant decrease in n. IX-X neuron number, the decline was greater in females (Fig. 2). Neuron numbers were essentially equal at stage 56 ( $p = .86$ ), but by stage 64 a sex difference in neuron number could be detected ( $p < .05$ ). Over this period, neuron number declined by 25% in males, but by 47% in females. The sex difference widened even further between stage 64 and adulthood, during which time male neuron number decreased by 35%, while female neuron number decreased by 42%. Overall, female neuron number declined by 69% from the peak stage 56 number,

while male numbers declined by 51%. Thus, sexual dimorphism in n. IX-X neuron number arises through a greater net loss of cells from females during tadpole and juvenile development.

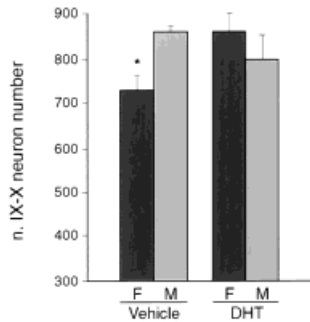
### Ontogenetic Cell Death Occurs during a Protracted Period of n. IX-X Development

We hypothesized that the greater net cell loss in females might reflect the survival of fewer female n. IX-X neurons during a period of naturally occurring cell death. The incidence of cell death in n. IX-X during development was not known, however, and it remained possible that the decreases in cell number we observed were due to other factors such as migration of cells out of the nucleus or specification of cells we identified as neurons to a nonneuronal fate. To demonstrate that cell death does in fact occur during n. IX-X development, we used TUNEL histochemistry to stain tissue for apoptotic nuclei.

Terminal transferase-mediated dUTP nick-end labeling-positive n. IX-X cells were found in both males and females at stage 56 (not shown), stage 64 [Fig. 3(A,C)], 1 week postmetamorphosis (not shown), and also in stage 54 animals of unknown sex (not shown). We also observed pyknotic nuclei in both males and females at stages 56 (not shown) and 64 [Fig. 3(B,D)], providing additional confirmation that cell death occurred at these stages. No TUNEL labeling or pyknosis was observed in adult n. IX-X cells. These results demonstrate that the ontogenetic death of n. IX-X neurons occurs over a period of at least 40 days, from stage 54 until, at minimum, the week after metamorphosis. This period of cell death coincides with decreases in the number of n. IX-X neurons (Fig. 2), indicating that the cell loss we observed in n. IX-X could be attributed, at least in part, to cell death.

### Hormonal Regulation of Sex Differences in Neuron Number

To investigate the ability of androgen to influence sexual differentiation of neuron number, we treated tadpoles with DHT or vehicle beginning at stage 54 and determined neuron numbers at stage 64 (Fig. 4). As expected, vehicle-treated males had significantly more n. IX-X neurons than vehicle-treated females ( $p < .02$ ); this finding confirmed our results from counts of untreated stage 64 animals. DHT treatment had no effect on neuron number in males (treated-male vs. vehicle-male;  $p = .25$ ), but treatment did significantly increase the number of neurons observed



**Figure 4** Counts of n. IX-X neurons in stage 64 tadpoles treated with either DHT or vehicle. M = male; F = female. \* $p < .02$ . In vehicle-treated animals, males had significantly more neurons than females, but in DHT-treated animals, the number of neurons did not differ significantly between the sexes. Neuron number in DHT-treated females also did not differ significantly from that of vehicle-treated males. Thus, DHT-treatment abolished the sex difference in neuron number normally observed at stage 64.

in females at stage 64 (treated-female vs. vehicle-female;  $p < .05$ ). This increase was sufficient to eliminate any significant difference between DHT females and DHT or vehicle-treated males ( $p = .39$  for both comparisons).

How does DHT treatment affect female neuron number? One interpretation is that DHT reduces the number of neurons that die between stages 56 and 64 to male-typical levels. Other explanations are possible, however—DHT might also stimulate the birth of additional n. IX-X neurons, or recruit newly born neurons originally destined for another location into n. IX-X. To investigate this possibility, we injected vehicle- or DHT-treated tadpoles with [ $^3$ H]thymidine and assessed the number of labeled cells in n. IX-X at stage 64. In keeping with previous observations (Gorlick and Kelley, 1987), we found very few [ $^3$ H]thymidine-labeled n. IX-X cells following our injection at stage 55–56, demonstrating that the vast majority of n. IX-X cells undergo final cell division prior to stage 55. No animal of either sex had more than four labeled n. IX-X cells, regardless of treatment group; most had no labeled cells ( $n = 5$  of each sex and each treatment). These results show that DHT treatment is unable to increase the number of n. IX-X neurons generated at stage 55–56. Thus, the DHT-induced increases in female neuron number we observed are likely not the result of androgen-mediated recruitment of new n. IX-X neurons.

We conclude that androgen treatment prevents the initial establishment of sex differences in n. IX-X neuron number, and that it does so by reducing the magnitude of the decrease in neuron number that would normally be observed in females by stage 64.

## Quantification of TUNEL-Positive n. IX-X Cells

Our cell count data suggest that sex-specific rates of cell loss between stages 56 and 64 may be responsible for sexual differentiation of n. IX-X neuron number. If true, then the number of dying cells ought to be higher in females during some or all of the 30-day period that separates stages 56 and 64. In an attempt to demonstrate sex differences in the number of dying cells, we counted TUNEL-positive n. IX-X cells in stage 56 and stage 64 animals. We found that the number of TUNEL-positive cells was small compared to the total number of neurons (as quantified in Cresyl violet-stained tissue) and did not differ systematically between the sexes. At stage 56, only ~2.1% of n. IX-X cells were TUNEL positive, indicating that cell death is not a widespread phenomenon in n. IX-X at this stage. Stage 56 males had  $40.8 \pm 12.3$  TUNEL-positive cells, while females had  $11.5 \pm 2.1$  (mean  $\pm$  S.E.M.;  $n = 4$  of each sex; not shown); these values did not differ significantly between the sexes ( $p = .25$ ). At stage 64, only ~7.3% of n. IX-X cells were TUNEL positive, indicating that cell death is not frequent in n. IX-X at this stage, either. Stage 64 males had  $60.6 \pm 7.4$  TUNEL-positive cells, while females had  $52.6 \pm 5.0$  (mean  $\pm$  S.E.M.;  $n = 4$  of each sex; not shown); again, these values were not significantly different ( $p = .35$ ).

Our failure to find sex differences in TUNEL-positive cells could indicate that the rate of cell death does not in fact differ between the sexes; it could also indicate that the sex difference in death rate occurs at or between stages 57 and 63. The temporal resolution of the TUNEL technique may also explain why no sex difference was detected (see Discussion).

## DISCUSSION

In this study, we sought to understand the developmental origins of a sexually dimorphic trait in the CNS component of the *Xenopus* vocal neuromuscular system. We have demonstrated that sex differences in n. IX-X neuron number first arise between stage 56 and 64. How is this sex difference established? We show that DHT treatment abolishes the sex difference at stage 64 by slowing the normal decline in female neuron numbers. This result suggests that androgens are involved in producing the sex difference in neuron number, possibly by regulating naturally occurring cell death of n. IX-X neurons.

## Androgen Regulation of Cell Death

Gonadal hormones have been shown to influence the rate of neuronal death during development of sexually dimorphic CNS nuclei in several different experimental systems (Konishi and Akutagawa, 1987; Kim and DeVogd, 1989; Sengelaub and Arnold, 1989; Davis et al., 1996). For example, in the rat spinal nucleus of the bulbocavernosus (SNB), a sexually dimorphic neuromuscular system in which sex differences in neuron number arise during a period of naturally occurring cell death, androgens can rescue neurons from death (Nordeen et al., 1985). Sex differences in neuron number are established by higher levels of androgen exposure in males and the resulting reduction in the number of cells lost during the period of developmental cell death (Sengelaub and Arnold, 1989; Jordan et al., 1997). Here, we present evidence for a similar scenario in the establishment of sex differences in laryngeal motor neuron number in *Xenopus*—we suggest that androgens can rescue n. IX-X neurons from naturally occurring cell death during development, and that this action is responsible for producing sex differences in n. IX-X neuron number in adulthood.

The first step in testing this hypothesis is to ask whether n. IX-X neurons undergo developmental cell death at all. We show that both sexes exhibit a net loss of over half of their n. IX-X neurons between stage 56 and adulthood, a scale of cell loss typical of motor nuclei that are subject to a period of naturally occurring cell death (Hughes, 1961; Oppenheim, 1991). TUNEL staining and identification of pyknotic nuclei confirm that neuronal death accompanies these reductions in neuron number, suggesting that the observed cell loss reflects a bona fide period of naturally occurring cell death. Is cell death linked to sexual differentiation of n. IX-X? We show that the cell death period coincides with the first appearance of sex differences in neuron number, suggesting that the two events may be related. The greater net loss of n. IX-X neurons in females observed over the course of the cell death period also indicates that the rate of cell death might be sexually dimorphic.

Are androgens responsible for establishing sexual dimorphism in the rate of cell death? We show here that when male and female tadpoles were exposed to exogenous DHT, the sex difference in n. IX-X neuron number normally observed at stage 64 failed to develop—the number of neurons in DHT-treated females at stage 64 was essentially identical to the number in untreated males. We also show that DHT produced this effect in females by preventing the loss of neurons prior to stage 64, rather than by stimulating

the addition of newborn neurons to n. IX-X to replace dead cells. This finding demonstrates that androgens are indeed capable of affecting the rate of cell death. Moreover, it shows that exposure to an identical androgenic hormonal environment equalizes the rate of cell death in males and females, suggesting that in normal development, differing levels of androgen exposure in males and females may be responsible for sex-specific rates of cell death. Taken together, our results provide good support for the notion that androgens effect sexual differentiation of n. IX-X neuron number by rescuing neurons from cell death—higher levels of androgens in males could result in a slower rate of death and more surviving neurons in adulthood.

## Do n. IX-X Neurons Die at Different Rates in Males and Females?

Naturally occurring neuronal death is usually considered to occur during a limited developmental period, at least in well-studied model systems (Oppenheim, 1991). In *Xenopus* lumbar motor neurons, for example, ontogenetic cell death occurs during stages 54 and 55, a period that lasts ~7–10 days (Hughes, 1961; Prestige, 1967). Our results demonstrate, however, that naturally occurring cell death in n. IX-X occurs over a much longer period—we observed TUNEL-positive n. IX-X cells at ages ranging from stage 54 to 1 week postmetamorphosis. These developmental stages are generally 40–50 days apart (Niewkoop and Faber, 1956; Hughes, 1961). Because developmental death of motor neurons in *Xenopus* has been shown to require thyroxine secretion, which starts at stage 54 (Prestige and Wilson, 1972), we consider it likely that stage 54 represents the beginning of the cell death period in n. IX-X. The end point of the cell death period, however, may be any time between 1 week postmetamorphosis and adulthood (in pilot studies, we never observed TUNEL labeling in adults). This is clearly a much more protracted period of developmental neuronal death than is usually observed in anuran, avian, or mammalian CNS nuclei (Kirn and DeVogd, 1989; Oppenheim, 1991).

The unusual length of the cell death period for n. IX-X neurons may explain one potential inconsistency in our results. According to our working model of n. IX-X sexual differentiation, androgens produce a sex difference in neuron number by slowing the rate of cell death in males. This implies that during the cell death period, males should have fewer dying n. IX-X neurons than females. We tried to demonstrate a lower rate of cell death in males by counting TUNEL-positive neurons in stage 56 and 64 tadpoles, but we

found no significant sex difference in the number of labeled cells. This result could mean that the rate of cell death is not in fact sexually dimorphic. However, we consider it more likely that the extraordinarily long period of cell death simply made the odds of finding a sex difference using the TUNEL technique extremely small. Apoptotic cells can be phagocytosed and cleared away in a matter of 1–2 h (Jacobson et al., 1997). TUNEL staining thus has a very limited temporal resolution in relation to the time over which we were trying to assess cell death. Since sexual differentiation of neuron number occurs over such a long period, the use of a technique that samples dying cells over such a short period is not appropriate, and is likely to yield inaccurate estimates of the rate of cell death.

Without a direct measurement of higher rates of cell death in females, the possibility remains that cell death is not the mechanism by which a dimorphism in n. IX-X neuron number arises. For example, ontogenetic cell death might remove a substantial but equal number of neurons in the two sexes, at the same time that androgens induce the migration of a smaller number of cells into the male n. IX-X. In this case, our cell counts would reveal a net loss of neurons from both sexes with a larger net loss in females, which is what we did in fact observe. DHT treatment would then raise female n. IX-X cell numbers at stage 64 by triggering migration into the nucleus rather than by preventing cell death. This scenario seems rather unlikely, however, because of the question of the origin of cells that could hypothetically be added to n. IX-X. Our [<sup>3</sup>H]thymidine experiment, as well as previous work (Gorlick and Kelley, 1987), indicates that neurons born at or after stage 55–56 do not normally integrate into n. IX-X. Nor are they induced to do so by DHT treatment. Thus, newly generated neurons are ruled out as a source of cells that could be summoned to n. IX-X in a sexually dimorphic manner between stages 56 and 64. The only possible source of additional n. IX-X cells would therefore be a pool of postmitotic neuronal precursors that could be induced to migrate into the nucleus upon exposure to androgen. We are not aware of any evidence that precursors such as these exist in *Xenopus*. However, we cannot rule out the possibility that migration of postmitotic cells into n. IX-X does contribute to sex differences in neuron number.

### Differential Effects of Androgen on Cell Survival and Axon Outgrowth

In previous studies, we used counts of the number of axons innervating the larynx to explore the possibility

that androgens influence survival of laryngeal motor neurons. We have shown that sex differences in laryngeal axon number arise between stages 56 and 62, and that androgen treatment abolishes this sex difference at stage 62 by increasing axon number in both males and females (Kelley and Dennison, 1990; Robertson et al., 1994). It was not clear from these studies, however, whether axon number is a reliable indicator of neuron number—androgens may exert their effects on axon number either by influencing the number of parent neurons, by influencing axon outgrowth or retraction, or by some combination of these processes. One important goal of the present study was to distinguish between these possibilities.

A comparison of laryngeal axon number and n. IX-X neuron number during development suggests that the axon loss observed by Kelley and Dennison (1990) likely does reflect the loss of cell bodies from n. IX-X. Axon number rises between stages 54 and 56 and then undergoes a substantial decline by adulthood in both sexes, a pattern we also observed for n. IX-X cell number. The net decline in axon number between stage 56 and adulthood is similar in scale to the net decline in neuron number—males lose 47% of their axons and 51% of their cell bodies over that time, while females lose 64% of their axons and 69% of their neurons. Neither axon number nor neuron number is sexually dimorphic at stage 56, but both become so over the subsequent ~3 weeks, a time when naturally occurring cell death takes place. Thus, the timing and scale of axon loss and neuron loss are quite similar, as is the timing of sexual differentiation of these traits. These results suggest that declines in axon number cannot be attributed solely to axon retraction or paring away of collateral axons—axon loss is accompanied by the loss of neurons from n. IX-X.

However, gains in axon number that result from DHT treatment do not appear to reflect rises in neuron number. Tadpoles of either sex treated between stages 54 and 62 with DHT showed significant increases in axon number over control males (Robertson et al., 1994). However, when we treated tadpoles with an identical dose of DHT between stages 54 and 64, we found that neither males nor females had more neurons than control males. Thus, DHT treatment can increase the number of laryngeal axons without producing an equal increase in the number of n. IX-X cell bodies. This finding indicates that DHT can induce addition of axons to the laryngeal nerve, either by causing collateral sprouting from axons already innervating the larynx or by increasing the number of n. IX-X cells that project there.

Together, our results suggest that androgens can affect the laryngeal motor neurons in two distinct

ways, each of which is important for the development of laryngeal innervation. One way is by regulating survival of these neurons—we show here that the loss of laryngeal axons during development is well correlated with the loss of n. IX-X cell bodies due to ontogenetic cell death, indicating that axon loss can be primarily explained by death of the parent neuron. The other way is by stimulating axon outgrowth— androgenic effects on this process probably account for the greater addition of axons to the male nerve that is observed during development (Kelley and Dennison, 1990).

### Mechanisms of Androgen Influence on Cell Survival

How is cell death in n. IX-X regulated during development? Our results suggest that androgens are involved; however, they do not appear to be central to the basic process of cell death. Androgens have only limited ability to influence the rate of cell death, as shown by the fact that exogenous DHT treatment cannot rescue additional n. IX-X neurons in males. Even though our dose of DHT was high enough to stimulate suprphysiological levels of laryngeal axon outgrowth (Robertson et al., 1994), it still did not prevent the loss of ~30% of n. IX-X neurons between stages 56 and 64. It appears, then, that androgens may function as weakly interacting modulators of the cell death program, capable of altering the loss of cells only enough to produce a sex difference in neuron number.

If androgens are only peripherally involved in regulating cell death, what are the main controlling factors? One likely candidate hormone is thyroxine. This hormone is secreted starting at stage 54 and is known to drive the process of metamorphosis (Leloup and Buscaglia, 1977; Dodd and Dodd, 1976). Interference with thyroxine function blocks the naturally occurring cell death of motor neurons throughout the *Xenopus* CNS, and it can also abolish the loss of laryngeal axons that occurs during late tadpole stages (Prestige and Wilson, 1972; Robertson and Kelley, 1996). These facts suggest that thyroxine secretion may initiate a common cell death program in all motor nuclei, including n. IX-X. In support of this notion, we observed TUNEL-positive cells in n. IX-X at stage 54, the beginning of thyroxine secretion and the time cell death begins in spinal motor neurons (Hughes, 1961; Prestige, 1967). We propose that androgens may modulate this thyroxine-controlled cell death program in n. IX-X neurons to bring about sexual differentiation of neuron number. Androgens have been shown to regulate the expression of cell-survival factors in ro-

dents (Al-Shamma and Arnold, 1997; Xu and Forger, 1998; Forger et al., 1998) and in *Xenopus* (Perez and Kelley 1997); such regulation provides a potential mechanism by which androgens might influence the cell death program.

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