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The Ontogeny of Androgen Receptors in the CNS of *Xenopus laevis* Frogs

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Androgenic steroids have been implicated in the development of sex differences in *Xenopus laevis* frogs. In order to determine when neurons first acquire the ability to concentrate androgen, we prepared autoradiograms of CNS in developing frogs following injection of tritiated dihydrotestosterone (DHT). *X. laevis* tadpoles and juveniles from stage 60 to 2 months post-metamorphosis (PM) were injected with [³H]DHT. Brain and spinal cord autoradiograms from these animals were examined for the presence of labelled cells. The pattern of [³H]DHT labelling in stage-64 tadpoles and in PM juveniles was similar but not identical to that seen in adults. Heavily labelled cells were seen in the motor nucleus of cranial nerves IX and X, medullary reticular formation, a presumed sensory nucleus of cranial nerve V, pretrigeminal nucleus of the dorsal tegmental area of the medulla, laminar nucleus of the torus semicircularis, anterior pituitary, ventral thalamus and anterior spinal cord. The vestibular sensory nucleus of cranial nerve VIII was the only area that concentrates DHT in adults but did not contain labelled cells in young animals. No [³H]DHT-labelled cells were found in stage-60 tadpoles. The onset of androgen concentrating capability in *X. laevis* CNS thus probably occurs between stages 60 and 64.

INTRODUCTION

The gonadal androgens, testosterone and DHT, are powerful mediators of male sex behavior^{3,17,22}. In South African clawed frogs, *Xenopus laevis*, castration of sexually active adult males eliminates both major sex behaviors, calling and clasping, while administration of testosterone or DHT to castrates restores both behavior patterns^{12,16,42}. Many behavioral effects of androgens are believed to be due to action on steroid-sensitive target areas in the brain^{3,17,22}. Autoradiographic studies have identified labelled cells in specific CNS nuclei after administration of radioactive testosterone¹⁵ and DHT¹³ to adult male and female *X. laevis*. These androgen-target nuclei include those participating in the control of male-specific sex behaviors^{13,42}.

We are interested in the developmental processes that mediate sexually dimorphic behavior in adults. Androgen affects the development of muscles³³ and motor neurons² that control male sex behaviors. We wish to determine how CNS areas implicated in the control of sex behaviors are affected by hormones

during development. Steroid hormones are presumed to act through cellular mechanisms that involve their selective accumulation and retention within cells²³. The present investigation used autoradiography to study the ontogeny of DHT concentrating capability in *X. laevis* CNS. A number of studies have used biochemical assays to determine the levels of androgen receptors in brain regions during development^{5,19,24,40,41}. However, only a few workers^{25,36} have used the more anatomically precise autoradiographic approach to investigate the ontogeny of androgen-concentrating neurons. Radiolabelled DHT was used here to identify cells containing androgen receptor. Unlike testosterone, DHT is not metabolized to estradiol in the brain^{22,31,32} and we can thus be confident that DHT-concentrating cells revealed by autoradiography are androgen-specific. To our knowledge, this is the first time autoradiography has been used to investigate the development of CNS androgen sensitivity over time. Determining (1) what CNS targets are sensitive to DHT during development and (2) when during development these targets first gain the ability to concentrate DHT, are

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necessary steps in understanding developmental mechanisms underlying neural control of male-specific behavior patterns.

MATERIALS AND METHODS

Animals

Twenty-four *Xenopus laevis* tadpoles and juveniles from stage 60 (ref. 27) to two months PM were used in this study. We examined 6 tadpoles at stage 60 (3 males, 3 females), 5 at stage 64/65 (3 males, 2 females), 3 female frogs at 2 weeks PM, 2 males and 2 females at 1 month PM and 3 males, 3 females at 2 months PM. Animals were obtained from Nasco (Ft. Atkinson, WI).

Administration of [³H]DHT

All of the PM animals were gonadectomized one week prior to [³H]DHT injection to clear any circulating androgen which might compete with labelled steroid. Sex was identified by visual inspection of the gonads in situ under a dissecting microscope. The suprarenal glands were removed from these animals at the time of sacrifice and were examined histologically for the presence of remnant or regenerated gonadal tissue. Premetamorphic frogs were not gonadectomized because their immature gonads are difficult to locate and, in pilot experiments, few survived surgery. All animals were injected with 5 μ Ci/g b. wt. of 1,2,4,5,6,7,16,17-³H]DHT (New England Nuclear, spec. act. 200 Ci/mM). Immediately prior to injection, the [³H]DHT was re-crystallized from benzene-ethanol and redissolved in a 1:1 solution of ethanol and 0.65% saline. PM and stage-64/65 animals were injected in the dorsal lymph sac and stage-60 tadpoles were injected i.p.

Autoradiography

Animals were allowed to survive for 2–2½ h after injection and were then deeply anesthetized with 0.25% MS-222 and chilled in crushed ice. Brains were quickly removed, frozen in powdered dry ice and mounted on cryostat chucks. The brains were then cut into 10- μ m horizontal sections in a Bright cryostat at –22 °C under safelight illumination. Every third section was picked up on Kodak NTB-2 nuclear track emulsion-coated slides.

Four additional animals (stage-48 tadpoles) were

freeze-mounted whole on chucks and 6 μ m transverse and horizontal sections through the entire body were prepared. Autoradiograms revealed some labelled cells in the head mesenchyme but cell localization was difficult due to poor tissue preservation. We therefore prepared 46 additional embryos and tadpoles from stages 11–64 by lightly fixing in 10% formalin and sinking in 15% sucrose before sectioning. While excellent morphology was obtained, we saw no labelled cells in the brains of fixed/sucrose-infiltrated stage-64 tadpoles; unfixed/uninfiltrated tadpole brains at stage 64 yielded autoradiograms with many labelled cells. No negative chemographic artifacts were apparent in fixed/sucrose-infiltrated sections. The failure to obtain expected labelled cells under fixation/sucrose infiltration suggests steroid loss from the tissue possibly due to receptor denaturation. Estradiol and testosterone steroid autoradiography has been successfully accomplished in rodents, however, with light aldehyde fixation and sucrose infiltration²⁶.

Sections were allowed to air dry in the dark and were then stored with desiccant in sealed, light-tight boxes at 4 °C. A third of the slides from each animal was exposed for 4, 6 or 8 months. Slides were developed for 1 min in Kodak D19 at 16 °C, rinsed in Kodak liquid hardener for 30 s, fixed in two changes of Kodak fixer for a total of 12 min and rinsed in tap water for 30 min. Brain autoradiograms were counterstained with cresyl violet. Whole-body autoradiograms were counterstained with hematoxylin-eosin.

Autoradiographic controls

Controls for positive and negative chemographic artifacts³⁰ were prepared from brain sections of a stage-60, a 2-week and a 2-month PM animal and from whole-body sections of a stage-48 tadpole. These animals were not injected with [³H]DHT but were processed for autoradiography at the same time as, and in an identical manner to, radioactive frog tissue. Non-radioactive tissue from all 4 control animals was picked up on emulsion-coated slides as controls for artifactual grain reduction and on light-exposed slides as controls for fading of the latent image. Neither chemographic effect occurred.

Analysis of autoradiograms

All sections were systematically scanned at 400×

with a Zeiss compound microscope for the presence of [^3H]DHT-labelled cells. A labelled cell was defined as one in which the number of reduced silver grains over the cell nucleus met or exceeded a criterion of $5\times$ background. In most cases, labelling was obvious by visual inspection. In the few instances when labelling was close to criterion levels, the number of reduced silver grains over the cell in question was compared with a background count obtained by averaging the number of grains in 5 adjacent cell-sized areas of neuropil. All brain sections containing labelled cells were drawn with the aid of a microprojector and the location of each labelled cell was indicated. The distribution and anatomical location of labelled cells were examined with the aid of these drawings and the neuroanatomical descriptions of Kelley¹³, Nikundiwe and Nieuwenhuys²⁸ and Simpson et al.³⁸.

RESULTS

Brain autoradiograms

Examination of autoradiograms revealed heavy [^3H]DHT labelled cells in specific nuclei of brains from all animals, stage-64/65 to 2-month PM (Table I). Labelled cells were observed in n. IX-X, medullary reticular formation, a presumed n.Vs, pretrigeminal nucleus of the DTAM, LTOR, APIT and VT (Fig. 1). The same areas were labelled in males and females. Gonadal tissue remnants with recognizable ova or spermatids were found in 3 animals (one male and one female 2-month-PM and in one 2-week-PM

TABLE I

[^3H]DHT labelled cells in the brain of *X. laevis* between stage 60 and 2 months PM.

-, no labelled cells; +, few labelled cells; ++, moderate number of labelled cells; +++, many labelled cells; 0, no data.

Nucleus	Stage 60	Stage 64/65	2 weeks PM	1 month PM	2 month PM
n. IX-X	-	+++	+++	+++	+++
Ri	-	++	++	+++	+++
VEST	-	-	-	-	-
n.Vs	-	+++	+++	+++	+++
DTAM	-	++	++	++	++
LTOR	-	++	++	++	++
APIT	-	+++	+++	0	+++
VT	-	+	+	+	+

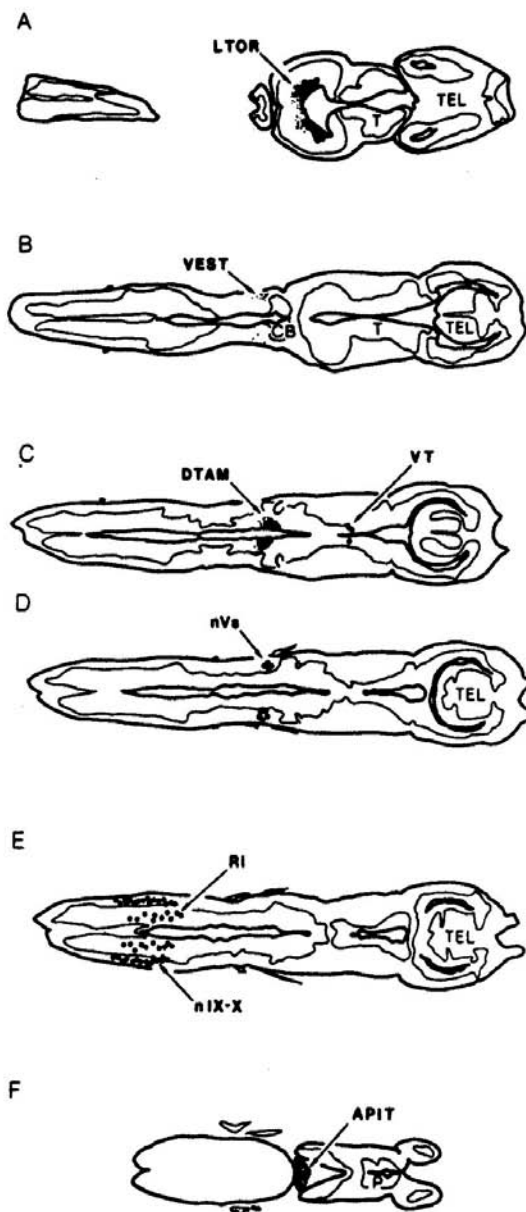


Fig. 1. Drawings of representative horizontal $10\text{-}\mu\text{m}$ sections through the brain of a stage 64-*X. laevis* tadpole. Anterior is to the right. Sections are presented in dorsal-to-ventral order. Cell groupings seen after cresyl violet staining are outlined. The locations of [^3H]DHT labelled cells in stage-64 to 2-month-PM animals are indicated by black dots. Shading indicates the extent of [^3H]DHT labelling in adult LTOR, VEST and DTAM. A: label in lateral part of laminar torus. B: no label in the vestibular sensory nucleus of cranial nerve VIII. C: sparse label in VT and label only in the anterior part of DTAM. Heavy label in a presumed n. V (D), n. IX-X and the Ri (E), and APIT (F).

female) when the animals were sacrificed for autoradiography. No difference in brain labelling was noted between these animals and those in which gonadectomy was complete. No label was seen in the brains of any of the stage-60 tadpoles (Table I). [^3H]DHT accumulation in stage-64/65 to 2-month-PM brains will be described in a caudal-rostral sequence.

Cranial nerve nucleus IX-X

Many heavily labelled motor neurons were seen in n. IX-X (Fig. 2). This nucleus extends posteriorly from the insertion of cranial nerves IX and X at the level of the obex until a point just rostral to the motor columns of the spinal cord. Labelled neurons occurred throughout the rostrocaudal extent of motor n. IX-X. Most of the cells in this nucleus are located in a long column in the white matter of the caudal medulla. Labelled motor neurons were also found in a

group of more anteromedial n. IX-X cells located at the lateral edge of the medullary grey.

Inferior reticular formation

Two groups of scattered labelled cells were seen in the medial caudal medulla. One group of cells was found immediately medial to the n. IX-X motor neurons in the lateral grey and the other group occupied a more dorsolateral position in the medial grey. These areas correspond to the dorsolateral and dorsomedial parts of the Ri^{28,43}, respectively. Many more labelled cells were seen in the reticular formation in 1- and 2-month-PM animals than were seen in stage 64/65 or 2-week-PM (Table I).

Sensory nuclei of cranial nerves

A compact group of labelled small cells was seen in the rostral medulla (Fig. 2). These cells are located in

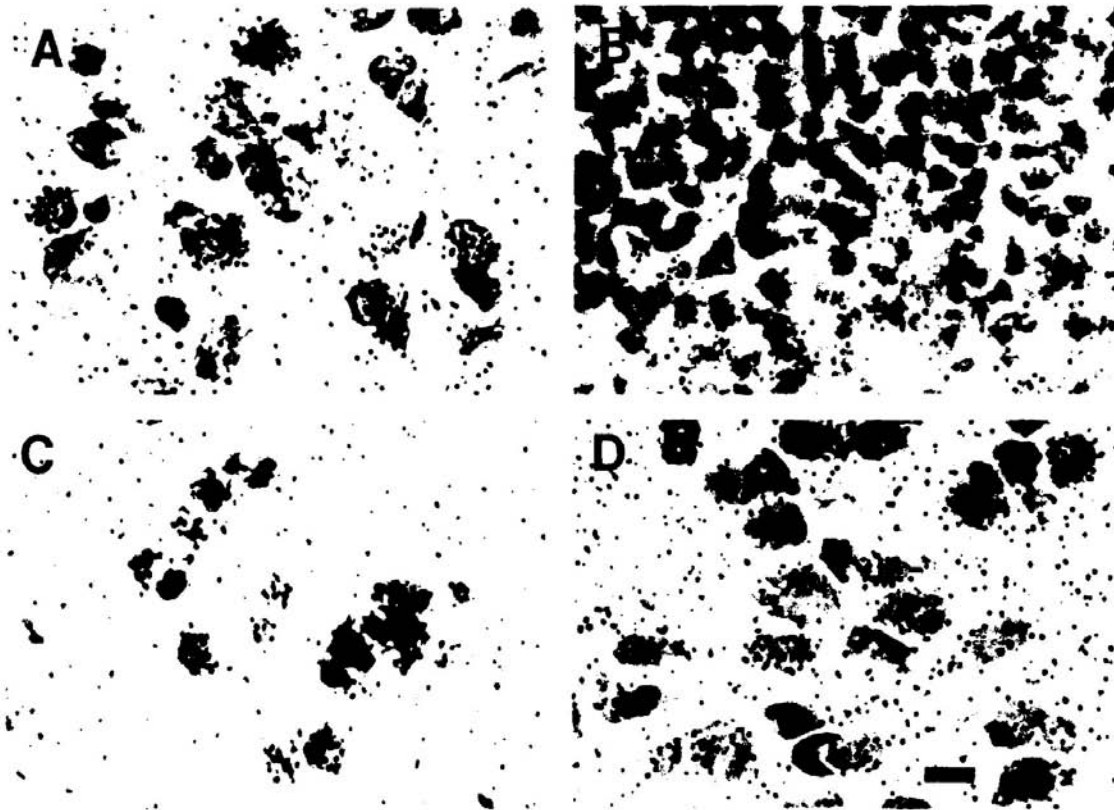


Fig. 2. Photomicrographs of autoradiograms of [^3H]DHT concentrating cells. Exposure time for all autoradiograms was 6 months. Cells are counterstained with cresyl violet. Scale bar is 10 μm and applies to all photomicrographs. A: motor neurons of cranial nerve n. IX-X in a 2-week-PM female. B: labelled APIT cells in a stage-64 male. C: labelled n. Vs cells in a 2-week-PM female. D: labelled DTAM cells in a 2-week-PM female.

the white matter, ventrocaudal and lateral to the motor nucleus of cranial n. V (Fig. 1), at the level of the entrance of cranial nerves VII and VIII. These cells probably correspond to the presumed sensory nucleus of cranial n. V^{13,28}, an area that concentrates [³H]DHT in adult *Xenopus laevis*. No labelled cells were ever seen in the area of the vestibular nucleus of cranial nerve VIII¹³, an area that also contains [³H]DHT-labelled cells in adults.

Dorsal tegmental area of the medulla

Some heavily labelled cells were consistently found in the anteromedial part of DTAM (Table I and Fig. 2). DTAM is located ventral to the cerebellum in the dorsal tegmentum. In horizontal section, this nucleus occupies a position lateral to the cerebral aqueduct and medial to the nucleus isthmi and the secondary visceral nucleus.

Laminar nucleus of the torus semicircularis

The torus semicircularis is a large mesencephalic structure located dorsal to the tegmentum that extends posteriorly from the tectal ventricle. The LTOR occupies the most rostral third of the torus and extends for 5 or 6 laminae caudal to the tectal ventricle. Some labelled cells were always found in the lateral portion of LTOR (Fig. 1).

Anterior pituitary

After [³H]DHT injection large numbers of heavily labelled cells were seen in the pars distalis of the pituitary (Fig. 2).

Ventral thalamus

Only a few labelled cells were seen in the thalamus and they did not occur in every animal; 3 cells were seen in a 2-month-PM animal, one in a 1-month-PM frog, one each in two 2-week-PM frogs and one each in two stage-64/65 tadpoles.

Labelling in the anterior spinal cord

Two–10 labelled large motor neurons were seen in the brachial enlargement of the spinal cord in every 2-week, 1-month- and 2-month-PM animal. Only one labelled brachial motor neuron was seen in one stage-64/65 tadpole. These motor neurons concentrate androgens in adults^{4,12} and have been implicated in the control of clasping by males during amplexus⁴. An

occasional labelled small cell was seen in the dorsal spinal grey and a few scattered labelled spinal motor neurons were seen caudal to the brachial enlargement.

DISCUSSION

Our results suggest that the onset of androgen concentrating ability in *X. laevis* CNS occurs just prior to metamorphosis. We found that the pattern of [³H]DHT labelling in stage-64/65 tadpoles and in PM juveniles was similar but not identical to that seen in adults. No [³H]DHT labelled cells were found in stage-60 tadpoles. Sensitivity to androgen thus most probably arises between stage 60 and stage 64.

Comparison of stage-64 and adult labelling pattern

Labelling in the motor nucleus of n. IX–X and in APIT of stage-64 tadpoles was identical to that seen in adult *Xenopus*¹³. There were, however, differences in either the number or location of labelled cells in all of the other DHT-sensitive areas. There were fewer labelled cells in the reticular formation at stage 64/65 and at 2 weeks PM than there were at 1 month and 2 months PM (see Table I). This was the only nucleus that showed a gradual increase to an adult number of labelled cells during the developmental period studied. Though adult-like numbers of labelled cells are evident in motor n. IX–X, APIT and the presumed n. Vs, the location of n. V is more lateral and caudal in the young animals studied here than in adults. Labelling in DTAM and LTOR follow a different pattern. The numbers of labelled cells in these areas do not change between stage 64 and 2 months PM, but remain at less than adult levels. However, labelling in both areas is restricted to only a part of the nucleus (see Fig. 1); DHT sensitivity in cells in the rest of DTAM and LTOR must develop later on. There were many fewer labelled cells in the ventral thalamus of the animals studied here than are found in adults, although even in adults this area does not have many labelled cells¹³. Increase in the number of DHT concentrating cells in this area must also occur later in development. Labelling in the ventral thalamus was extremely sparse in this study. A total of only 8 labelled cells were seen in 6 animals out of the 24 animals examined. In contrast, all 10 adults examined by Kelley¹³ had labelled cells in this area.

A striking difference in our results was the absence of labelling in the vestibular sensory nucleus of cranial nerve VIII, an area that contains numerous [³H]DHT concentrating cells in adults¹³. That this area was the only one showing a complete lack of labelling up to 2 months PM raises the possibility that androgen sensitivity here arises very late in development.

Onset of androgen sensitivity

All stage-64/65 tadpoles had many heavily labelled cells in androgen-target brain nuclei. None of the stage-60 tadpole brains exhibited any DHT-concentrating neurons. The most straightforward interpretation of this result is that androgen-target neurons first develop the capacity to accumulate detectable quantities of DHT at some point between stage 60 and stage 64. It is possible, however, that we did not detect any labelled cells in stage-60 animals because of competition from endogenous androgen. Neither stage-60 nor stage-64 tadpoles were gonadectomized. Yet stage-64 tadpoles had many labelled cells indicating that the mere presence of the testes is not sufficient to preclude labelling. One might argue that stage-60 tadpoles are producing very high levels of androgen, higher than those seen in stage-64 tadpoles or in PM frogs whose gonadectomies were not complete (both of the latter groups had many DHT-labelled cells). We are currently examining androgen titers at this time in development. It is, of course, possible that stage 60 tadpoles display low levels of androgen receptor which cannot be detected using autoradiography. Cytosolic assays reveal a low level of estrogen receptors in whole fetal mouse brain one to two days before receptors can be visualized in specific brain nuclei by autoradiographic methods^{6,11}. We also considered the possibility that the intraperitoneal injection route (stage 60) is inferior to the dorsal lymph sac (stage 64 and older) in making hormone available to the CNS. However, intraperitoneal injections in *Rana pipiens* yield many androgen-concentrating cells¹⁸.

Comparison with rodents

Autoradiographic studies of the ontogeny of hormone receptors in the CNS have been primarily conducted on rodents. Most of these investigations are single time-point assays of the location of hormone

concentrating cells. The pattern of testosterone-concentrating cells and estradiol-concentrating cells in two-day-old female rat brain^{35,36,37} and of estrogen concentrating cells in 16-day-old fetal mouse CNS³⁹ is similar to that in adults. Two-week-old male chick brain also shows adult-like [³H]testosterone labelling²⁵. Our results agree with these in that we saw labelling approximating the adult pattern in stage-64/65 tadpoles and in juvenile frogs.

Longer term autoradiographic studies of the development of hormone-concentrating capability have been limited to estrogen^{6,11,21}. A recent paper by Keefer and Holderegger¹¹ described the ontogeny of estrogen receptors in mice. Their results with [³H]estradiol parallel our results with [³H]DHT in many ways. They found that estrogen-containing cells appeared in most brain areas late in development (embryonic day 14) and that the number of labelled cells in most areas reached adult levels by embryonic day 17. Four out of 14 brain nuclei remained at very low subadult levels, however, and two areas showed no labelling at birth. We found that in *X. laevis*, [³H]DHT concentrating cells also appear late in embryonic development (between stage 60 and 64) and that the number of labelled cells quickly reaches adult levels in most nuclei. Numbers remain low only in the ventral thalamus while one nucleus (vestibular of VIII) never shows labelling by two months PM. It thus appears that the overall ontogenetic pattern of estrogen sensitivity in mouse brain and of androgen sensitivity in *Xenopus* frog brain is quite similar; both arise rapidly during the later stages of embryonic development.

Development of hormone sensitivity

In all vertebrates examined to date, steroid hormone-concentrating cells in CNS represent a restricted subset of brain and spinal cord neurons. The genetic and/or ontogenetic factors responsible for the expression of steroid receptors in these particular cells are unknown. One possibility is that the appearance of receptors in androgen target neurons is a completely hormone-independent developmental event. A second possibility is that steroid target neurons first begin to express low levels of receptor at very early stages of development (possibly before the final mitosis of the neuron's 'birthday') but that exposure to hormone at a particular developmental stage

upregulates receptors to the point where they become detectable using autoradiographic or biochemical methods. The latter notion is congruent with recent findings on the development of sexually dimorphic telencephalic nuclei in zebra finch brain. Adult male zebra finches have more androgen-concentrating neurons in certain brain nuclei than do females¹. Male chicks display a rise in estradiol levels at hatching¹⁰. Exposure of females to estradiol shortly after hatching leads to a male-like number of androgen-concentrating neurons when females are exposed to androgen as adults²⁹. A parallel regime of hormone treatment (early estrogen, late androgen) also causes females to engage in an otherwise exclusively male behavior, song⁸.

Unlike the telencephalic androgen targets of zebra finches, there is no gross sexual dimorphism in the percentage of androgen-concentrating neurons in adult *Xenopus laevis* brain^{13,15}. As reported here, the sexes also do not differ in their developmental history of expression of androgen receptors. Even the laryngeal motor neuron nucleus, whose cells in adults are highly dimorphic in number⁹ and in dendritic extent¹⁴ appears to have equivalent proportions of [³H]DHT-accumulating neurons in both sexes of developing and adult frogs. Biochemical measurements of androgen receptor levels in laryngeal muscle, the target of these motor neurons, reveal that adult males have 3–4× more receptor than do adult females³⁴. Adult female levels are not upregulated to that of males by androgen treatment³⁴ suggesting that sex differences in receptor level of adult *X. laevis* do not reflect sex differences in circulating androgens but are instead the permanent result of a developmental pat-

tern of hormone secretion. In *X. laevis* CNS, however, androgen-concentrating neurons may upregulate receptor levels between stages 60 and 64 in response to endogenous hormone secretions.

Critical periods in sexual differentiation

One characteristic of CNS sexual differentiation is the occurrence of critical or sensitive periods during development²⁰ when exposure to hormone permanently alters the responsiveness of steroid target tissues. The opening of such critical periods could be set either by the first appearance of receptor in the cells or by the beginnings of steroid secretion from the gonads. In rodents, androgen and estradiol receptors in hypothalamus and preoptic area cells first appear shortly after these neurons are 'born'^{11,40}. This is also when blood levels of both hormones are beginning to rise⁴⁰, suggesting that the machinery for the steroid response may be in place just prior to its activation. In *X. laevis*, many of the DHT-concentrating neurons (in n. IX–X, DTAM, VT and Ri) become PM at stages 50–56⁸, shortly before [³H]DHT concentrating ability appears. Although the testes appear histologically competent to produce hormone by stage 59 (ref. 27), we do not know when secretion begins. Further speculation on the mechanism underlying the ontogeny of [³H]DHT sensitivity in *X. laevis* thus awaits radioimmunoassay determination of serum levels of androgen during development.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

APIT	anterior pituitary
CB	cerebellum
CNS	central nervous system
DHT	dihydrotestosterone
DTAM	dorsal tegmental area of the medulla
LTOR	laminar nucleus of the torus semicircularis
n. Vs	sensory nucleus of cranial nerve V

n. IX–X	motor nucleus of cranial nerves IX and X
P	preoptic area
PM	postmetamorphosis
Ri	inferior reticular formation
T	thalamus
TEL	telencephalon
VEST	vestibular nucleus of cranial nerve VIII
VT	ventral thalamus

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