Proc. Natl. Acad. Sci. USA
Vol. 78, No. 9, pp. 5876–5880, September 1981
Neurobiology

Modulation of the neural control of the clasp reflex in male *Xenopus laevis* by androgens: A multidisciplinary study

(steroids/spinal neurons/testosterone 5α-reductase)

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Communicated by George B. Koelle, May 11, 1981

ABSTRACT **The neural control of the clasp reflex in male *Xenopus laevis* has been studied by using anatomical, electrophysiological, and biochemical techniques. Neurons in spinal segment 2 of castrated males accumulate label after injection of [^{3}H]-dihydrotestosterone; these neurons are distributed within the rostral portions of the motoneuronal pools of the sternoradialis and flexor carpi radialis muscles. *In vitro* recordings from the nerve to the sternoradialis muscle in the isolated spinal cord preparation from castrated male *Xenopus* showed increased activation to paired dorsal root stimulation after addition of dihydrotestosterone to the bath. This increase could be prevented by prior administration of cycloheximide. The reducing enzyme testosterone 5α-reductase is present and is selectively distributed in male *Xenopus* spinal cord. It is speculated that the androgens may alter patterns of neuronal activity leading to the "clasp" muscles and thereby influence myosin types within these muscles.**

The clasp reflex in frogs is an important component of sexual behavior. The reflex is manifested by the sexually active male grasping the receptive female around the waist with his front legs and orienting himself into a pelvic clasp position, which may be maintained for many hours. This behavior can be induced in normal male *Xenopus laevis* by administration of chorionic gonadotropin, and in castrated animals by implantation of pellets of testosterone or of dihydrotestosterone (H_2testosterone) in the lymph sacs (1). Several neural areas related to sexual activity have been identified in the male (2, 3), and the results have shown that the neural pathways for the clasp reflex in Anura are present in the medulla and spinal cord. Autoradiographic studies (4) using [^{3}H]H_2testosterone have shown that the label can be taken up by nuclei of some neurons of spinal segments 2 and 3 of *X. laevis*. These segments contain neurons that receive inputs via the dorsal roots from the skin of the pads, inner surfaces of the forearm, and chest; when these cutaneous surfaces are stimulated, the strength of the clasp is modified. These segments also contain motoneurons that project to the muscles of the forelimbs responsible for clasp behavior.

These observations raise questions concerning the mechanisms by which hormones may modify the neural control of sexual behavior. We have used the isolated spinal cord of the frog as a model to study the mechanisms by a multidisciplinary approach and to determine how H_2testosterone may influence the activity of spinal neurons projecting to the muscles primarily involved in the clasp reflex. We have sought first to identify the loci of neurons innervating these muscles and to determine whether overlap in location exists with those neurons that concentrate H_2testosterone. Second, we have investigated the electrophysiological consequences of such steroid uptake. Finally, in order to assess whether testosterone might act through its metabolite H_2testosterone, we looked for the presence of its converting enzyme, testosterone 5α-reductase, in these same spinal regions. The changes in neuronal behavior elicited by administration of androgen may reflect those that occur normally with changes in endogenous androgen levels during the sexual cycle of the animal.

Some of these results have been reported in abstract form (5–7).

METHODS

In all experiments, adult male *Xenopus laevis* were used.

Anatomic Studies. The location of spinal cord motoneurons innervating the primary muscles subserving the clasp reflex (8, 9), the sternoradialis ($n = 4$) and flexor carpi radialis ($n = 4$), was determined by using the horseradish peroxidase (HRP) technique. Adult male *X. laevis* were anesthetized (Finquel, Ayerst Laboratories), and the muscles were exposed and injected with 3 μl of 4% HRP (Sigma, type VI) in frog Ringer's solution. After 3 days the animal was anesthetized and transcardially perfused. The spinal cord was removed, placed in fresh fixative, washed, and stored overnight in a 30% sucrose/phosphate buffer solution at 4°C. Frozen sections were cut at 50 μm. HRP activity was histochemically demonstrated, using tetramethylbenzidine as the chromagen (10); sections were lightly stained with neutral red and microscopically examined for the presence of HRP-labeled cells.

The location of androgen accumulation was determined autoradiographically, using the nonaromatizable androgen H_2testosterone. Castrated adult males ($n = 5$) were injected with 200 μCi (1 Ci = 3.7×10^{10} becquerels) of octatritiated H_2testosterone (300 ng of H_2testosterone, New England Nuclear). After a 2-hr survival period, the spinal cord was removed, frozen, and processed for autoradiography (4). Slides were exposed for 4, 6, or 8 weeks, developed, lightly stained with cresyl violet acetate, and microscopically examined for the presence of labeled cells. Cells were judged to be labeled when the number of silver grains over the cell nucleus equaled or exceeded five times the number of grains over adjacent nucleus-sized areas of neuropil.

Electrophysiological Procedures. After transection of the brain at the tectum the nerve to the sternoradialis muscle was exposed ventrally, dissected free, and followed into the spinal nerve. The dorsal surface of the cord was then exposed and all roots except the dorsal and ventral roots of segment 2 were cut.

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Abbreviations: H_2testosterone, dihydrotestosterone; HRP, horseradish peroxidase; DR, dorsal root.

The spinal cord was transected rostrally at the level of the entry of cranial nerve X and caudally at segment 7, removed, and transferred to a chamber containing normal Ringer's solution. The ventral root leading to the spinal nerve of segment 2 and thence to the nerve to the sternoradialis was led through a seal to a side chamber containing mineral oil and placed on silver or platinum hook electrodes. The dorsal root (DR) was cut at the ganglion and placed in a suction electrode. Nerve responses were recorded through Grass P15 ac preamplifiers that relayed first to a Tektronix 5113 storage oscilloscope and thence to Tektronix 5A10 and 3A3 amplifiers. The amplified signals were displayed on a Tektronix 565 oscilloscope and photographed. Intracellular recordings were obtained by using 2.6 M potassium citrate microelectrodes leading to a WPI 701 amplifier and thence to 3A10 amplifiers and the 565 oscilloscope. WPI 830 series waveform and pulse generators leading to 850 series stimulus isolation units were used for stimulation of DRs or the nerve to sternoradialis, or for intracellular current injection.

Biochemical Analysis. In order to assay for testosterone 5α-reductase activity, the whole spinal cord or segments of interest were removed from the animal while it was immersed in normal Ringer's solution at 12°C. The tissue was homogenized in 6 times its volume at 0°C. The homogenization buffer contained 0.1 M Na_2HPO_4 (pH 7.4), 1 μM dimethyl sulfoxide, 3.5 μl of glucose-6-phosphate dehydrogenase (1 unit/μl, Sigma), 2.2 μM unlabeled testosterone (Sigma), 4.6 mM NADP, and 2.4 mM glucose 6-phosphate. The final incubation volume of 70 μl consisted of 50 μl of the homogenization buffer and 20 μl of solution containing [^{3}H]testosterone solution; this was prepared by evaporating 35 μl (200 ng) of [1α,2α-(N)^{3}H]testosterone (53 Ci/mmol, Amersham) to dryness with nitrogen and redissolving in 300 μl of 0.1 M Na_2HPO_4 and 20 μl of dimethyl sulfoxide. Incubations were run for 3 hr in covered glass tubes in a shaking waterbath at 20°C. Reactions were terminated with 100 μl of ethanol/acetone (1:1, vol/vol). The reaction mixture was then centrifuged for 1 min in an Eppendorf–Brinkmann 5412 centrifuge at 15,000 rpm. Twenty microliters of the supernatant was removed and applied to thin-layer chromatographic sheets for separation and identification of ^{3}H-labeled steroids (11). Gelman ITLC-SA thin-layer sheets (Fisher) were coated with a solution of 20% (vol/vol) propylene glycol in acetone. The mobile phase was carbon tetrachloride/hexanes (9:1, vol/vol). Steroid standards were included and visualized with a *p*-anisaldehyde reagent. Each channel of the thin-layer plate was cut into 0.5-cm segments. Each segment was then immersed in scintillation fluid (formula 963, New England Nuclear) and the radioactivity was determined in a Searle Isocap 300 liquid scintillation counter. Protein was assayed by the method of Lowry *et al.* (12).

RESULTS

Distribution of Motoneurons that Concentrate ^{3}H-Labeled Androgen and Innervate Muscles Subserving the Clasp Reflex. The brachial enlargement is a 3.5-mm-long region that includes segments 2 and 3 and contains the lateral motor columns. HRP-labeled cells projecting to either the sternoradialis or flexor carpi radialis muscles were distributed rostrally in this region. The motoneurons that projected to the sternoradialis muscle were limited to the most rostral 1.7 mm of the enlargement and were located, on the average, more medially than those projecting to flexor carpi radialis.

Cells concentrating [^{3}H]H_2testosterone were observed exclusively through the rostral 0.5 mm of the brachial enlargement. This distribution of cells labeled with [^{3}H]H_2testosterone and the distribution of HRP-labeled cells are compared in Fig. 1. Motoneurons projecting to flexor carpi radialis had a similar location to the more laterally located cells that concentrated

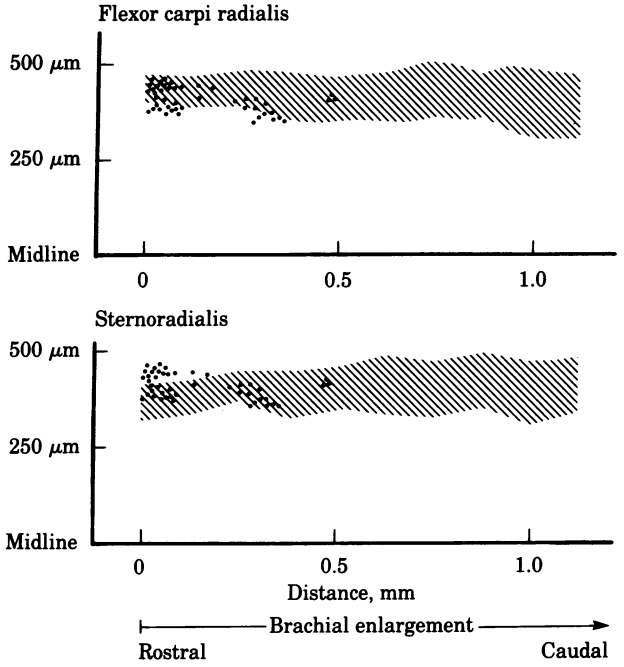

FIG. 1. Schematic horizontal sections through the brachial enlargement of *X. laevis* spinal cord. The locations of neurons innervating forelimb muscles are indicated as hatched areas; androgen-concentrating cells are represented by dots. (*Upper*) The more laterally located steroid-concentrating neurons overlap in position with the locations of ventral horn cells innervating the flexor carpi radialis muscle. (*Lower*) Medial steroid-labeled cells overlap with the generally more medial cells innervating the sternoradialis muscle. The motoneuron pool innervating these arm muscles includes the most rostral 1.7 mm of the enlargement; androgen-concentrating neurons are confined to the rostral 0.5 mm of this region.

[^{3}H]H_2testosterone, whereas those projecting to the sternoradialis had a similar location to the medially located ^{3}H-labeled cells. Examples of neurons in nucleus sternoradialis labeled with HRP and of neurons that concentrate [^{3}H]H_2testosterone are shown in Fig. 2 *A* and *B*, respectively.

Thus, a subset of neurons located within the motoneuronal pools that innervate sternoradialis and flexor carpi radialis muscles concentrate exogenously administered androgen in their nuclei. Nuclear accumulation of steroid is in keeping with the hypothesis that H_2testosterone influences the activity of target cells via a transcriptional process (13).

Effects of Steroids on the Activity of Spinal Neurons. The effects of the steroids on neuronal activity were tested on the isolated spinal cord preparation of male *Xenopus* that had been castrated 8–51 days previous to the recording. Fig. 3*A–C* shows three consecutive recordings from the nerve to the sternoradialis muscle to paired DR stimulation (interstimulus interval, 20 msec) after equilibration of the preparation in normal Ringer's solution for 2 hr at 12°C. The response to the second stimulus (S_2) is considerably greater (facilitation) than when S_2 is given alone (Fig. 3*D*). Fig. 3 *E–G* shows consecutive recordings obtained 3 hr after the addition of H_2testosterone (1 μM). The pattern of facilitation changed: it showed higher levels of synchronization and amplitude of the potential in response to S_2 than were seen in the absence of the steroid. Fig. 4 shows the response to S_2 (as reflected by the area under the compound action potential) before and 3 hr after administration of H_2testosterone as a function of the interstimulus interval. The activation is significantly greater for intervals up to (and in some experiments, beyond) 800 msec. These results suggest that

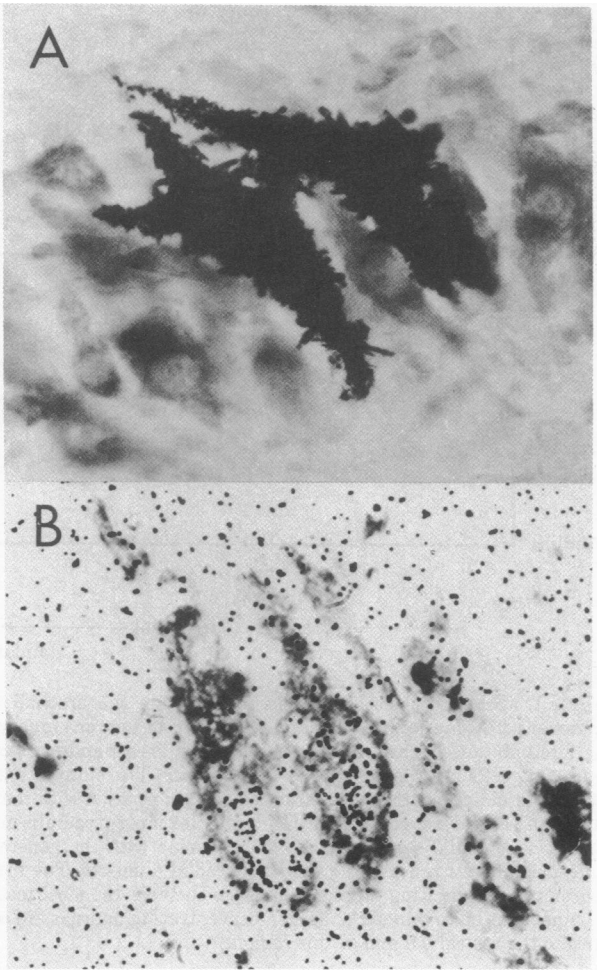

FIG. 2. Photomicrographs of brachial enlargement motoneurons of male *X. laevis*. (*A*) Neurons containing HRP after retrograde transport from the sternoradialis muscle. (Tetramethylbenzidine section counterstained with neutral red. ×750.) (*B*) Autoradiogram of labeled lateral motor column neurons in the brachial enlargement of a male *X. laevis* after systemic injection of [^{3}H]H_2testosterone. The section was lightly counterstained with cresyl violet acetate. Note the accumulation of silver grains over the unstained cell nucleus. (×750.)

H_2testosterone can (*i*) change the *patterns* of response of sternoradialis neurons to DR stimulation, and (*ii*) increase the *numbers* of neurons activated to firing threshold by DR stimulation by increasing either the membrane excitability or the probability for multiple spike firing.

The increased response to the H_2testosterone was manifested first at approximately 2 hr after its administration to the bath. In nonneural cells, androgenic steroids manifest their effects through a sequence of steps intracellularly, including nuclear binding (13). Autoradiographic studies (4, 14) have shown that after administration of tritiated testosterone or H_2testosterone to male *Xenopus*, label is concentrated in neuronal nuclei. We wondered whether the delay in the response to H_2testosterone resulted from a change in protein synthesis due to steroid-mediated changes in nuclear transcription. We therefore administered the protein synthesis inhibitor cycloheximide to the bath in concentrations ranging between 2 and 60 μg/ml. Cycloheximide alone had virtually no effect on the response to DR stimulation over a 2-hr period; when H_2testosterone was then added to the bath with cycloheximide, concentrations of the inhibitor above 30 μg/ml were effective in preventing the H_2testosterone-induced changes for at least an additional 7 hr.

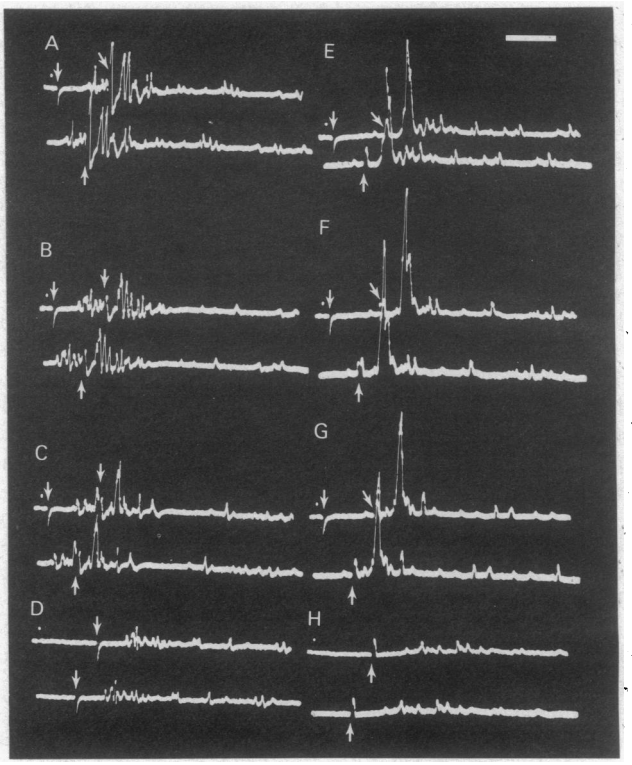

FIG. 3. Effects of H_2testosterone on spinal neuronal activity. Male *X. laevis* were castrated 50 days prior to recording. The recordings are from the nerve to the sternoradialis muscle in response to paired stimulation of the dorsal root of segment 2. In each recording, the top trace shows the responses to paired DR stimulation (arrows); the lower trace shows the same recording as the top trace but with the response to the second stimulus (at arrow) displaced to the left to show any later activity. (*A–C*) Consecutive recordings in normal Ringer's solution. (*D*) Response to single DR stimulation in normal Ringer's solution. (*E–G*) Consecutive recordings obtained 3 hr after addition of H_2testosterone to the perfusing Ringer's solution. (*H*) Response to single DR stimulus after addition of H_2testosterone to the Ringer's solution. Calibration point at the left of each top tracing indicates 50 μV; time line indicates 20 msec.

As the first step towards defining changes in neuronal membrane properties elicited by the androgen, we tried to record intracellularly in isolated spinal cords from sternoradialis mo-

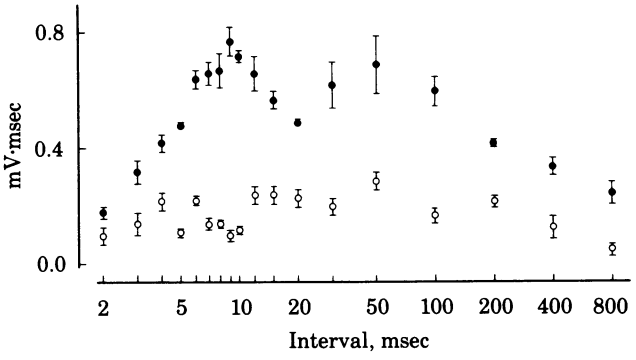

FIG. 4. Effects of H_2testosterone on spinal neuronal activity. Same experiment whose recordings (at 20-msec interstimulus interval) are shown in Fig. 3. The area under the compound action potential from the nerve to the sternoradialis muscle (±SEM) in response to the second DR stimulus is plotted against interstimulus interval. ○, Responses in normal Ringer's solution; ●, responses 3 hr after addition of H_2testosterone to the perfusing medium.

toneurons before and for at least 3 hr after addition of the steroid to the bath. We have not, however, succeeded in maintaining the electrode inside the cell during this procedure. Therefore, we compared patterns of responses obtained intracellularly from sternoradialis motoneurons from males induced (by prior chorionic gonadotropin injection) to clasp receptive females with the response patterns of sternoradialis motoneurons from castrated males. Gonadotropin injection into intact males increases circulating androgen levels; castration greatly reduces androgen titers (4). We have recorded intracellularly from 80 sternoradialis motoneurons from castrated males (castrated 12–52 days prior to recording) and 48 motoneurons from clasping males. Fig. 5 *A* and *B* shows responses to DR and to antidromic stimulation, respectively, of a sternoradialis motoneuron in the spinal cord of a male castrated 40 days prior to recording. Note the long latency of the responses and the single elicited spike. The spike showed AB configuration and was often generated from the descending part of a small excitatory postsynaptic potential. These responses are to be contrasted to those shown in Fig. 5 *C* and *D* recorded from a spinal motoneuron of a clasping adult male. DR stimulation elicited multiple spikes generated from long-lasting excitatory postsynaptic potentials (Fig. 5*C*). In some cases (not shown) monosynaptically elicited spikes were evoked by DR stimulation. Antidromic stimulation also elicited multiple spike firing (Fig. 5*D*). These different response patterns were characteristic of but not exclusive to the two groups of animals. We have rarely (twice) recorded the response patterns of Fig. 5*A* from motoneurons of normal clasping males; yet this response was seen in 23 motoneurons of castrated males.

Two points must be emphasized: first, we do not know that the electrodes recorded from neurons that accumulated the hormone; and, second, the results depend upon sampling by the electrodes, so that the majority of cells from which records were obtained were probably large. These preliminary results suggest that patterns of activation of some neurons projecting to the sternoradialis muscle—a muscle primarily involved in clasping behavior—are strongly and relatively rapidly affected by androgenic steroids.

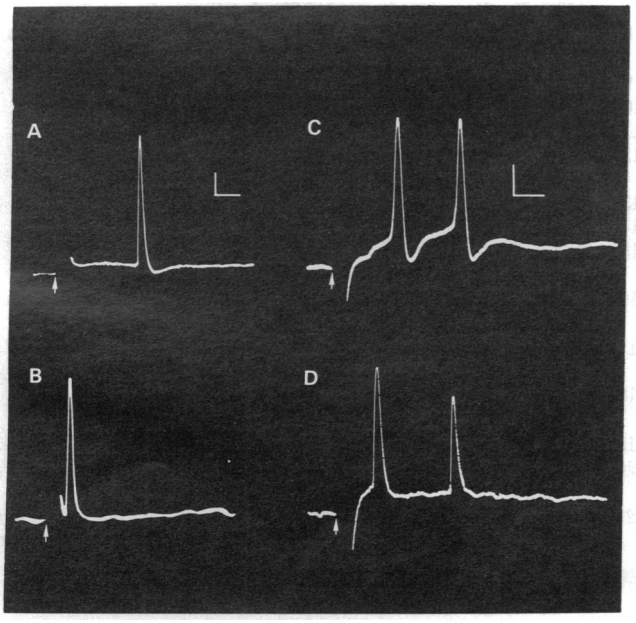

FIG. 5. Intracellularly recorded responses from sternoradialis motoneurons in *Xenopus laevis*. (*A* and *B*) Responses to single stimulus to DR (*A*) and sternoradialis nerve (*B*) stimulation from a spinal cord of a male castrated 40 days prior to recording. Calibration: 10 mV, 10 msec. (*C* and *D*) Responses to single stimulus to DR (*C*) and sternoradialis nerve (*D*) stimulation from a spinal cord of a clasping male. Calibration: 20 mV, 5 msec.

Presence of Testosterone 5α-Reductase in the Spinal Cord. The results of the above experiments are compatible with the hypothesis that both testosterone and H_2testosterone may be active compounds inducing sexual behavior in *X. laevis*. Both substances can induce clasping behavior in castrated male *Xenopus* when administered by subcutaneous or intralymphatic implantation, both substances are concentrated by central neurons, and both substances cause changes in firing patterns of spinal neurons. If testosterone acts by conversion to H_2testosterone, the testosterone-reducing enzyme might be present in the spinal cord. We have, therefore, examined testosterone 5α-reductase activity in the spinal cord and mapped the distribution of this enzyme in different spinal segments.

Segments of the spinal cord from normal males were homogenized and incubated with tritiated testosterone. Fig. 6 compares the chromatographic pattern for a 3-hr incubation of homogenate at 20°C to that for a boiled homogenate. The amount of H_2testosterone produced is proportional to the difference in areas under the two H_2testosterone peaks. Production of H_2testosterone with respect to time and protein concentration was linear. The K_m was 20 μM and the V_{max} was 10 pmol/mg protein per hr.

Fig. 7 shows that spinal segments 2 and 3 (those segments related to clasping behavior) have substantial 5α-reductase ac-

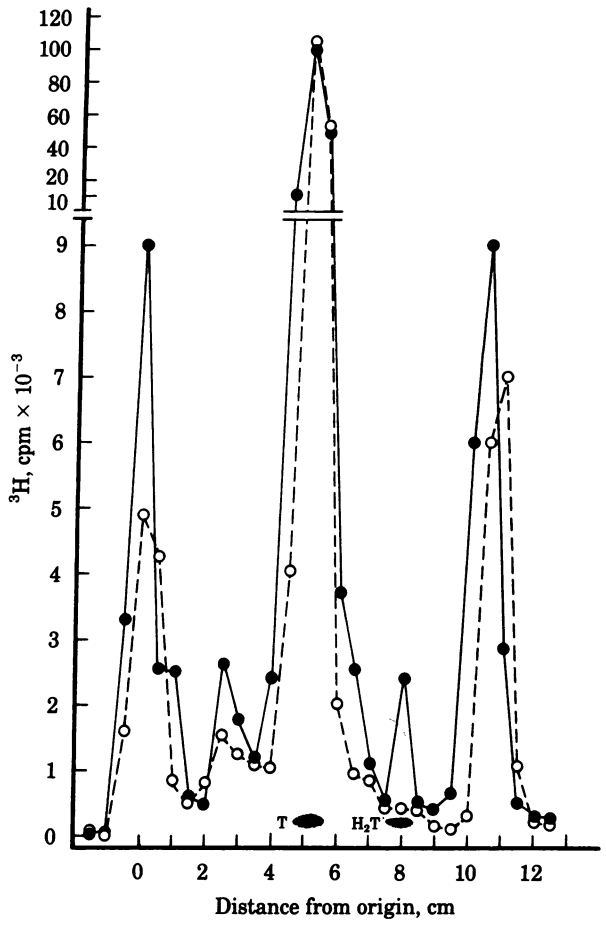

FIG. 6. Thin-layer chromatographic separation of tritiated steroids demonstrating 5α-reductase activity in homogenates of frog spinal cord. Tissue was incubated for 3 hr at 20°C in the presence of [^{3}H]testosterone. ●, Fresh tissue; ○, boiled tissue. T and H_2T indicate positions of testosterone and H_2testosterone.

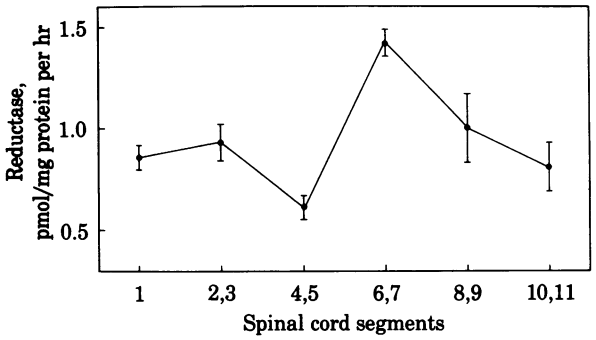

FIG. 7. Segmental distribution of testosterone 5α-reductase activity in *Xenopus* spinal cord. The data are expressed as mean values ± SEM; $n = 4$.

tivity; on the other hand, segments 4 and 5, whose neurons do not concentrate the hormone, have relatively low concentrations of enzyme activity. The lumbar areas of the cord (segments 6–9) also have considerable enzyme activity. Three conclusions arise from these experiments: (*i*) testosterone 5α-reductase is present in the frog spinal cord; (*ii*) the presence of enzyme activity suggests that testosterone could function in eliciting the motor behavior through conversion to H_2testosterone; and (*iii*) the distribution of testosterone 5α-reductase, as of H_2testosterone uptake, is not uniform but may correspond to the loci of neurons involved in sexual behavior.

DISCUSSION

The isolated spinal cord of *X. laevis* can be used to investigate the effects of androgenic steroids on neuronal activity. The advantage of the preparation lies in its suitability for multidisciplinary investigation, the diverse results of which can be related to the intact animal's behavior.

In a spinal cord preparation taken from a castrated male, H_2testosterone added to the perfusing medium changes the patterns of activation of neurons that project to the sternoradialis muscle, a primary muscle in clasping behavior. The change in the pattern of activity elicited by paired DR stimulation is prevented by cycloheximide administration, suggesting that H_2testosterone exerts its effects via protein synthesis. The neurons involved are presumably those that concentrate H_2testosterone in their nuclei (see ref. 4); these include neurons that are located within the sites of distribution of the motoneuronal nuclei of the sternoradialis and flexor carpi radialis muscles. Both interneurons and motoneurons may be involved.

Our studies do not resolve the question whether testosterone or H_2testosterone is the naturally active hormone in male *X. laevis*. The presence of testosterone 5α-reductase within the spinal cord, however, suggests that in the intact male testosterone is capable of being reduced to its active metabolite. We cannot exclude an alternative pathway whereby testosterone may be aromatized to estradiol, especially in view of the recent findings by Kelley *et al.* (15) and by Callard *et al.* (16, 17) that androgen may be aromatized to estrogen in frog brain and in cultured turtle brain cells. Our results to date, however, suggest that estradiol has no effect on patterns of activation of sternoradialis motoneurons. It is therefore still uncertain whether multiple pathways for the formation of active testosterone metabolites are present in amphibian spinal cord.

Hormonal–neuronal interactions, therefore, occur at specific neurons of the spinal cord of male *Xenopus*. In parallel studies (unpublished) it has been shown by the use of antibodies specific to fast and slow myosins that the primary clasp muscles contain both fast twitch and slow tonic muscle fibers. The fiber types within the muscle appear to depend upon the level of androgen present. Weeds *et al.* (18) have shown that, in fast- and slow-twitch muscle fibers of the cat, cross-innervation changes the myosin type; Salmons and Streter (19) have concluded on the basis of their studies that this effect may result from a change in nerve activity. Although androgens can exert direct effects on muscle (20, 21), we speculate that increased androgen levels during breeding season alter the firing patterns of the neurons projecting to the clasp muscles, in time causing a change in their myosin type. This would result in an increased ratio of slow tonic to fast twitch muscle fibers facilitating maintained clasp behavior in the sexually active male.

We are grateful to Drs. Alan Goldman, Jerry Strauss, and Linda Heffner for valuable advice throughout this study and to Dr. Alan Fine for constructive comments on the manuscript. We also thank Marie Griffin, Barbara Goun, Ken Anderson, and Anthony Riccio for excellent technical assistance. We acknowledge Grants NS 12211 and HD 12126 from the U.S. Public Health Service.

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