

Estrogen and Laryngeal Synaptic Strength in *Xenopus laevis*: Opposite Effects of Acute and Chronic Exposure

Kwok Hang Wu Martha L. Tobias Darcy B. Kelley

Department of Biological Sciences, Columbia University, New York, N.Y., USA

Key Words

Gonadal steroids • Synaptic strength • Larynx. Vocal behavior. Gonadotropins • Sexual receptivity. *Xenopus laevis* • Gonadectomy • Electrophysiology

Abstract

Synaptic transmission at the vocal synapse, the laryngeal neuromuscular junction, of *Xenopus laevis* has been shown to be regulated by long-term changes in circulating estrogen. In females, high levels of circulating estrogen also accompany gonadotropin-induced ovulation and oviposition and the switch from sexually unreceptive to receptive states, including changes in vocal behaviors (ticking to rapping). Here we examine the effects of gonadotropin injection on laryngeal synaptic strength and call type. Gonadotropin acutely reduced quantal content values of laryngeal synapses in intact, adult females; the lowest values were attained by 12 h post-injection. Estrogen and progesterone levels increased following human chorionic gonadotropin (hCG) injection; the time course was similar to, but negatively correlated with, changes in synaptic strength. In ovariectomized frogs, exogenous estrogen, but not progesterone or hCG, mimicked the acute effects of hCG in weakening laryngeal synapses of intact frogs. hCG injection suppressed ticking and sometimes induced rapping. Females could tick with either strong or weakened laryngeal synapses while rapping was only produced during

the weakening action of hCG. The normally strong synapses of females may enable vocal production even when laryngeal synapses are weakened by hormones that induce ovulation. In contrast to the acute effect of estrogen on weakening laryngeal synapses, juveniles required more than 2 weeks of estrogen treatment to strengthen laryngeal synapses while at least 4 weeks postovariectomy were required to weaken synapses in adult females. We conclude that acute (hours) increases in circulating levels of estrogen weaken synapses while chronic (weeks) increases strengthen laryngeal synapses.

Copyright © 2001 S. Karger AG, Basel

Introduction

In vertebrates, courtship and other reproductive behaviors are regulated by gonadal hormones. This regulation is achieved by hormone actions on cell targets, neural and muscular, which effect these behaviors. One example is courtship song in the South African clawed frog, *Xenopus laevis*. In this species, male and female specific courtship songs signal sexual identity, reproductive state and location [1]. Sex differences in strength of the vocal neuromuscular synapse contribute to male/female differences in song characteristics, such as the reliability of click production in females and click amplitude modulation in males [2–4].

The laryngeal synapse is estrogen sensitive; females have stronger synapses than males and synaptic strength relies on estrogen exposure during development [5, 6]. Thus, juveniles of both sexes have similarly weak synapses; males maintain weak synapses throughout development while synaptic strength in females increases in response to rising titers of estrogen. Exogenous estrogen treatment strengthens juvenile laryngeal synapses in both sexes while ovariectomy in immature females prevents synaptic strengthening as adults. The ovaries are also required for the maintenance of strong synapses; ovariectomy of adults results in synapse weakening. The duration of treatment used in producing these effects was long, 1.5–3 months. However, gonadal hormone levels rise rapidly in response to gonadotropin injection. In this paper we examine the effect of acute increase in gonadal hormone levels, following gonadotropin injection, on laryngeal synaptic strength. Hormone replacement in ovariectomized females was used to determine which ovarian hormone is responsible for gonadotropin-induced changes in synaptic strength. In addition, the minimum duration of estrogen manipulation required to increase synaptic strength in juveniles and to decrease synaptic strength following ovariectomy in adults was determined.

Female *X. laevis* produce distinct types of calls depending on sexual state [7]. Sexually unreceptive females usually tick, while sexually receptive females usually rap. Rapping is faster and louder than ticking. Here we consider the possibility that a brief rise in estrogen, associated with induction of sexual receptivity, affects laryngeal synaptic strength. Estrogen-induced changes in synaptic strength might be associated with call type; specifically, we predicted that synaptic strength would increase with receptivity and that stronger synapses would be associated with rapping. Receptivity can be reliably and rapidly induced by gonadotropin injection [7, 8]. In these experiments, we examined vocal behavior changes following gonadotropin injection and determined how these changes relate to laryngeal synaptic strength.

Materials and Methods

Animals

Adult females were purchased from Xenopus I (Ann Arbor, Mich., USA) or from Nasco (Ft. Atkinson, Wisc., USA). Juveniles, six months after completion of metamorphosis and weighing between five and ten grams, were purchased from Nasco. Animals were maintained in polycarbonate tanks containing dechlorinated water, on a 12:12 light:dark cycle (lights on at 08:00 and off at 20:00h), and fed frog brittle (Nasco) 3 times a week.

Endocrine Manipulation

Gonadectomy. For ovariectomy of adult females, animals were anesthetized by immersion in 0.12% MS222 (ethyl *m* amino benzoate methane sulfonic acid; Sigma, St. Louis, Mo., USA) in phosphate buffer (pH 7.4) and a small incision through the skin and muscles was made to one side of the abdominal midline. Ovaries and associated fat bodies were gently retracted and severed by electrocautery. The incisions were then sutured and the animal was allowed to recover in moist towels. A similar gonadectomy procedure was used for both male and female juveniles except that the juveniles were anesthetized in 0.09% MS222.

Gonadotropin Administration. For hCG (human chorionic gonadotropin) injection, 1 ml of hCG (1,000 IU; Sigma) was injected into the subcutaneous space above the thigh 6, 9, 12, 15, 18 or 48 h before the experiment. For each female, vocal behavior, laryngeal quantal content and circulating hormone titers were determined.

Steroid Hormone Administration. Estrogen implants were prepared by mixing a known amount of estradiol powder (β -estradiol; Sigma) with Silastic scalant (Dow Corning, Midland, Mich., USA): the mixture was used to fill a 1-ml syringe and extruded to form a narrow rod. When dry, the rod was weighed and cut into small blocks for implantation. The amount of estradiol in each block was estimated assuming homogenous distribution of estradiol within the block. Progesterone Silastic blocks were prepared in a similar way. For juveniles, estradiol Silastic blocks were implanted into the dorsal lymph sac via a small incision at the time of gonadectomy. The estradiol dosage was either 100–150 or 30–70 $\mu\text{g/g}$ body wt (in two different sets of experiments). The first dose is based on that used by Tobias and Kelley [5] while the second yields circulating concentrations in the physiological range. For gonadectomized adult females, estradiol-containing Silastic blocks were implanted under local anesthesia (10% Lidocaine, Sigma, in absolute ethanol was applied to the skin 1 min before incision) into the dorsal lymph sac 12 h before larynx removal. The adult dosage for estradiol was $\sim 20 \mu\text{g/g}$ and for progesterone (Sigma) was ~ 20 or $\sim 5 \mu\text{g/g}$ in two different sets of experiments. For progesterone, the lower dose yields circulating levels closer to those of hCG injected adult females; both doses give similar quantal content values. Frogs were deeply anesthetized by immersion in 0.15% MS222; blood was then collected transcardially for radioimmunoassay of hormone levels and the larynx was removed for quantal content measurements.

Electrophysiology

Quantal Content Measurements. As in previous studies in larynx [4, 5], quantal contents were determined using the failure analysis described by Del Castillo and Katz [9]. We did not compare spontaneous (miniature) to evoked end-plate potential amplitudes because mcpp frequency ($\sim 2/\text{min}$) is so low; stable recordings for more than 1 h would be required for measuring quantal content using amplitude. This limitation raises the possibility that some small amplitude mepp were treated as failures. We regard this possibility as unlikely because very small amplitude mepps ($\sim 1 \text{ mV}$) were reliably recorded. Further, the quality of recordings did not differ between experimental groups so that even if some mepps were not detected, the failure to detect should apply to all groups and would thus not bias comparisons of group means.

The procedures for measuring quantal content values are summarized briefly as follows. The larynx was removed from an anesthetized animal and pinned dorsal side up in a Sylgard (Dow Corning) coated Petri dish filled with a low calcium, high magnesium frog

saline (105 mM NaCl, 2 mM KCl, 0.5 mM CaCl₂, 5.0 mM MgCl₂, 27.7 mM dextrose and 4 mM Hepes buffer). Surrounding tissues and fat were then removed. The integrity of the laryngeal nerve was confirmed by stimulation via a suction electrode and subsequent laryngeal muscle contractions. The preparation was then allowed to equilibrate in the low calcium saline. After muscle contraction in response to nerve stimulation was blocked (2-3 h), intracellular responses were recorded. Muscle fibers were impaled with glass microelectrodes (20–50 MΩ) filled with 2.5 M potassium acetate and the nerve was stimulated with 0.5 ms electrical pulses at 3 s intervals. Muscle endplate recordings were amplified and acquired by a Macintosh computer via MacLab (ADInstruments Pty Ltd., Castle Hill, Australia). For each muscle fiber, the number of failures to induce EPSPs in response to 100 stimuli delivered to the nerve was determined. Quantal content (m) was then calculated: $m = \log_e(\text{number of trials} / \text{number of failures})$. For each animal, three to six muscle endplates were examined and the arithmetic mean of their quantal content values was obtained to yield a representative quantal content for the animal. The mean is a good measure of the central tendency of quantal contents of muscle endplates since mean and median were very close in value for all animals tested.

Mepp Amplitudes. Mepp frequency is very low under physiological conditions (about 0.03 Hz, or 2/min) and thus saline with high sucrose (0.125 M) was used to increase mepp frequency to 0.6–1.0 Hz. Quantal contents were first determined in low calcium saline and the saline was then replaced with a high sucrose/low calcium saline to determine mepp amplitude. Muscle fibers were impaled and electrical activity amplified and acquired by a Macintosh computer using MacLab. The amplitudes of 40–60 mepps were obtained for each muscle fiber; 5–7 fibers were examined from each animal. Using this procedure, quantal content values and mepp amplitudes were determined in different muscle fibers from the same animal.

Circulating Hormone Levels

All hormone concentrations were determined by solid-phase ¹²⁵I-radioimmunoassay with COAT-A-COUNT kits from Diagnostic Products Corporation (Los Angeles, Calif., USA). Blood from adult females was collected via an incision in the auricle. Blood from juveniles was collected via a capillary tube inserted into the ventricle; blood was extracted with suction. The blood samples were then allowed to clot on ice, spun to recover the serum and transferred to Eppendorf tubes and stored at –20°C until hormone assay. For estrogen, we measured levels of estradiol-17β (E₂), the major functional estrogen in *Xenopus* [10]. Serum concentrations of estradiol, progesterone, and hCG were measured with the estradiol kit (TKE21), progesterone kit (TKPG 1) and hCG IRMA kit (LKCG 1), respectively, according to the manufacturer's instructions.

Behavioral Studies

Because *X. laevis* vocalizes under water, calls were detected and recorded using a hydrophone (model H505L; Wilcoxon, Gaithersburg, Md., USA) in a 31 x 41 x 61 cm glass aquarium; the water depth was 21 cm and the water temperature was ~20°C. All behavioral tests were performed between September and December. Sexual activity in the stimulus male was induced by injection of 1,000 IU hCG at least 2 days before the test and 100 IU on each subsequent day. Females were injected with 1,000 IU hCG 6–48 h before larynx removal (performed as described above). On the day of testing, the male and female were introduced to the testing room at 15:00 h; the room was illuminated only by a dim red light. Tests for male/female vocal interactions started

at 17:00 h, the male was placed into a perforated, round, plastic container within the aquarium and the female was placed outside of the container. The container prevented physical contact but allowed vocal communication, a condition that increases the incidence of female rapping. Rapping can be discriminated by its fast click rate (13 Hz) and its ability to evoke prolonged male calling; ticking is a slower (4 Hz) call that silences males [7]. Vocal behaviors were taped (model PMD221; Marantz, Chatsworth, Calif., USA), and subsequently digitized and analyzed on a Macintosh Power PC using the Canary program (Correll Bioacoustics Laboratory; Ithaca, N.Y., USA). Interclick intervals of female calls were analyzed to confirm call type. If the female did not rap in the first 30 min, the container was removed to permit male clasp attempts. If the female was unreceptive, ticking was induced by male clasp attempts. Ticking and vocal interactions were monitored, recorded for 30 min and analyzed as described above. Ticking can be induced at any time by a clasping male; most observations of gonadectomized females were carried out in the afternoon. Female responses to male clasp attempts were monitored to assess sexual receptivity; unreceptive females extend their hind limbs and attempt to dislodge the male while sexually receptive females are quiescent and exhibit exaggerated knee flexion [8]. If the female was silent, vocalizations were recorded until 18:00 h at which time the female was anesthetized, blood was collected and the larynx was removed for quantal analysis.

Statistics

An analysis of variance (ANOVA) was used to assess differences between multiple groups (StatView software; SAS Institute, Cary, NC., USA). When a significant difference was present, a post-hoc Dunnett's test was performed [11, pp 194–195]. When only two groups were compared, the Student's t-test was employed (StatView software). The relation of two parameters was assessed by calculating correlation coefficients (StatView software); Fisher's r to z transformation was performed to detect the significance of correlations. When parametric methods were not possible, i.e. hCG levels in control animals were under detectable levels and no variance data were available for analysis, nonparametric methods were used. The tests used were: Kruskal-Wallis ANOVA test with post-hoc nonparametric Dunn-type multiple comparisons of treatments versus control [11, pp 200–201], and Spearman's rank correlation test [11, p 318]. All data are given as mean ± SEM (sample size). The significant level for all tests was set at 5%. The term 'control group' refers to the zero time treatment group in all endocrine experiments.

Results

hCG Injection Decreases Laryngeal Synaptic Strength: The Decrease Is Presynaptic

To determine how gonadotropin affects synaptic strength, adult females were injected with hCG and quantal contents measured between 6 and 48 h later. Figure 1a shows some representative electrophysiological recordings for quantal content determination. Laryngeal synaptic strength decreases after hCG injection (fig. 1b; $p = 0.0021$). By 12 h after injection, quantal content values are significantly lower than control values [0.38 ± 0.06 (6) vs. 1.49 ± 0.18 (9); $p < 0.01$] and remain depressed at

15 h [0.61 ± 0.16 (3); $p < 0.05$]. Quantal content values gradually recover and reach pre-injection levels by 48 h [1.58 ± 0.18 (3)]. We conclude that hCG administration produces an acute reduction in laryngeal synaptic strength.

The method of failures relies on the ability of nerve stimulation to evoke changes in membrane potential in muscle fibers; differences could be either pre- or postsynaptic in origin [12]. For example, higher failure rates following hCG administration might reflect a decrease in the postsynaptic response to acetylcholine at laryngeal synapses rather than decreases in transmitter release from the presynaptic terminal. To examine this possibility, mepp amplitudes (a measure of quantal size reflecting postsynaptic sensitivity) were compared in control frogs and in females 12 h after hCG injection. Mean mepp amplitudes from the two groups of animals do not differ [control: 0.88 ± 0.14 mV (3); hCG injected: 0.82 ± 0.09 mV (3); $p = 0.7001$ nor are mepp amplitudes and quantal contents determined from the same animals correlated ($r = -0.175$, $n = 6$, $p = 0.759$). These results suggest that the change in synaptic strength induced by hCG injection is not due to a change in the postsynaptic response of laryngeal synapses but instead reflects a change in neurotransmitter release from presynaptic terminals.

KG Injection Changes Vocal Behavior

Vocal behaviors produced by females when paired with an advertising male were recorded at various times after hCG injection (fig. 2). All uninjected females were sexually unreceptive and ticked. Gonadotropin-induced sexual receptivity was accompanied by suppression of ticking and, sometimes, the occurrence of rapping. Between 6 and 24 h after hCG injection, ticking was suppressed in all females. The duration of ticking suppression varied with the duration of oviposition. At 48 h after injection, one female ticked and had no retained eggs in her abdomen or oviduct while the other two were silent and retained eggs in their oviducts. The latter two females were still receptive as judged by their response to the clasping males. Between 6 and 24 h after hCG injection, some females were silent; all of these females were receptive when clasped by a male. Thus, silence in intact females is an indicator of sexual receptivity. Between 9 and 18 h after hCG injection, some females rapped. No female rapped without hCG injection but not all sexually receptive females rapped; of the 21 receptive females tested, 5 rapped. Although rapping is difficult to elicit in the laboratory, it is readily produced by wild-caught receptive females in a more natural environment [7].

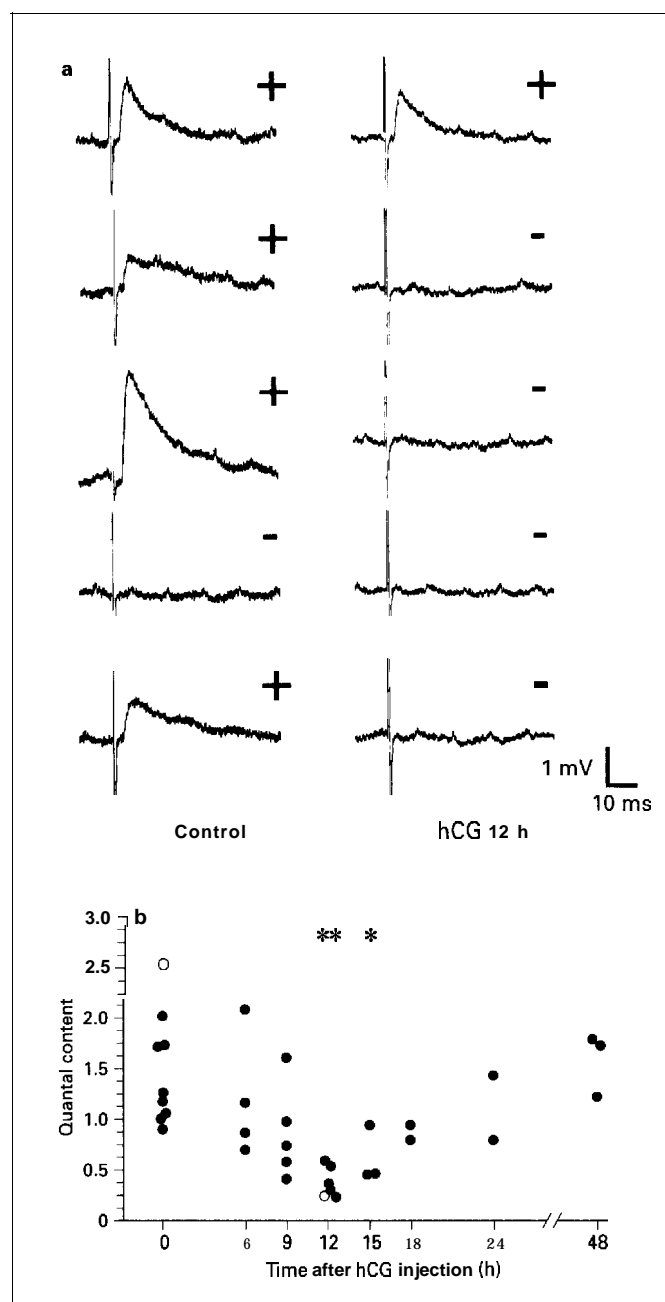


Fig. 1. Quantal content measurement in laryngeal synapses after hCG treatment. **a** Representative electrophysiological recordings from one control (left) and one hCG-injected (right) female muscle fiber. Five consecutive recordings from the same cell are shown. Failures are denoted with ‘-’ signs, and epsps are marked with ‘+’ signs. The average quantal contents of the control (0 h) and hCG-treated (12 h) cells illustrated in (a) are denoted by the lighter dots in part b. **b** Quantal contents at various times after hCG injection. Individual data points indicate the averages of quantal contents from 3 to 6 fibers from the same animal. Quantal content decreases after hCG injection, reaches a minimum at 12 h (** $p < 0.01$) and remains at a significantly lower level at 15 h (* $p < 0.05$). Synaptic strength then increases and reaches control levels by 48 h.

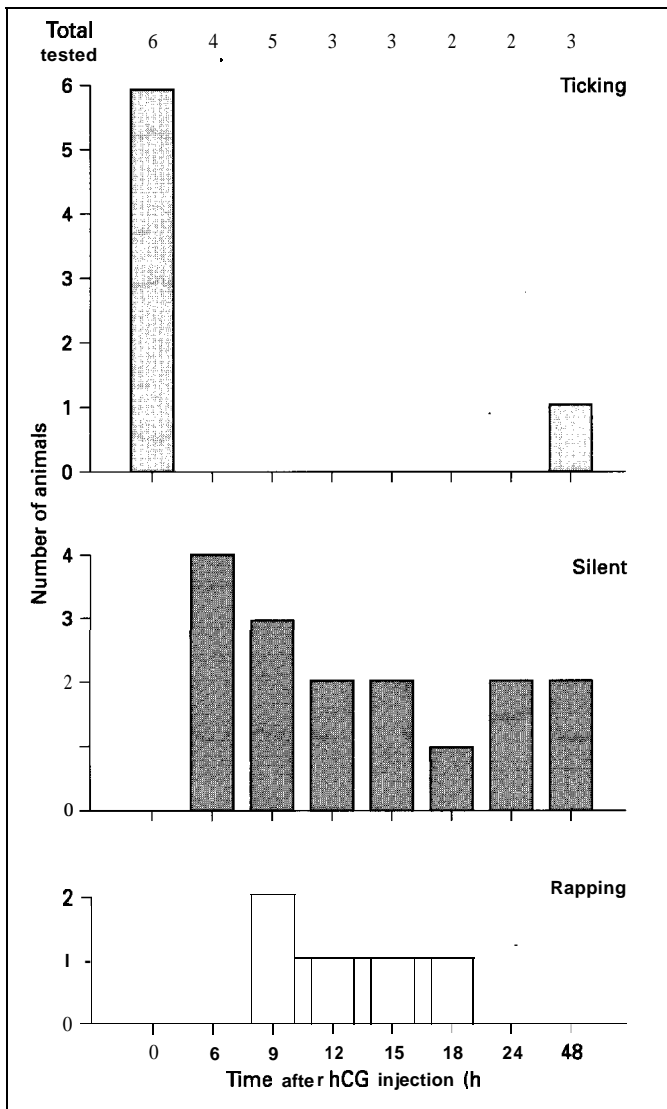


Fig. 2. Female vocal behaviors after hCG injection. Number of females exhibiting each vocal behavior between 0 and 48 h after hCG injection. Ticking is the call of unreceptive females and all uninjected females ticked. After hCG injection, animals became sexually receptive and ticking was suppressed; females either rapped or were silent. Rapping was produced between 9 and 18 h after hCG injection.

Gonadal Steroid Level Increases as Laryngeal Synaptic Strength Decreases

The data described above reveal an acute depression of laryngeal synapses by hCG, known to stimulate estrogen and progesterone secretion. However, rising levels of endogenous gonadal steroids during development, as well as exogenous estrogen treatment, induce strong laryngeal synapses [5, 6]. We thus examined steroid levels in hCG-injected females and compared them to synaptic strength.

Estradiol (E2) levels increase significantly after hCG injection (fig. 3a; $p = 0.0007$). The mean E2 level in females without hCG treatment is 901 ± 237 (9) pg/ml (3.31 ± 0.87 nM). In hCG-injected females, E2 levels increase after hCG injection and are significantly higher than control values at 12 h [$10,735 \pm 2,646$ (6) pg/ml, $p < 0.011$]. Maximum E2 values are obtained 15 h after hCG injection [$12,333 \pm 2,914$ (3) pg/ml, $p < 0.011$]. Subsequently, E2 levels decrease but remain higher than control values for as long as 2 days after hCG injection.

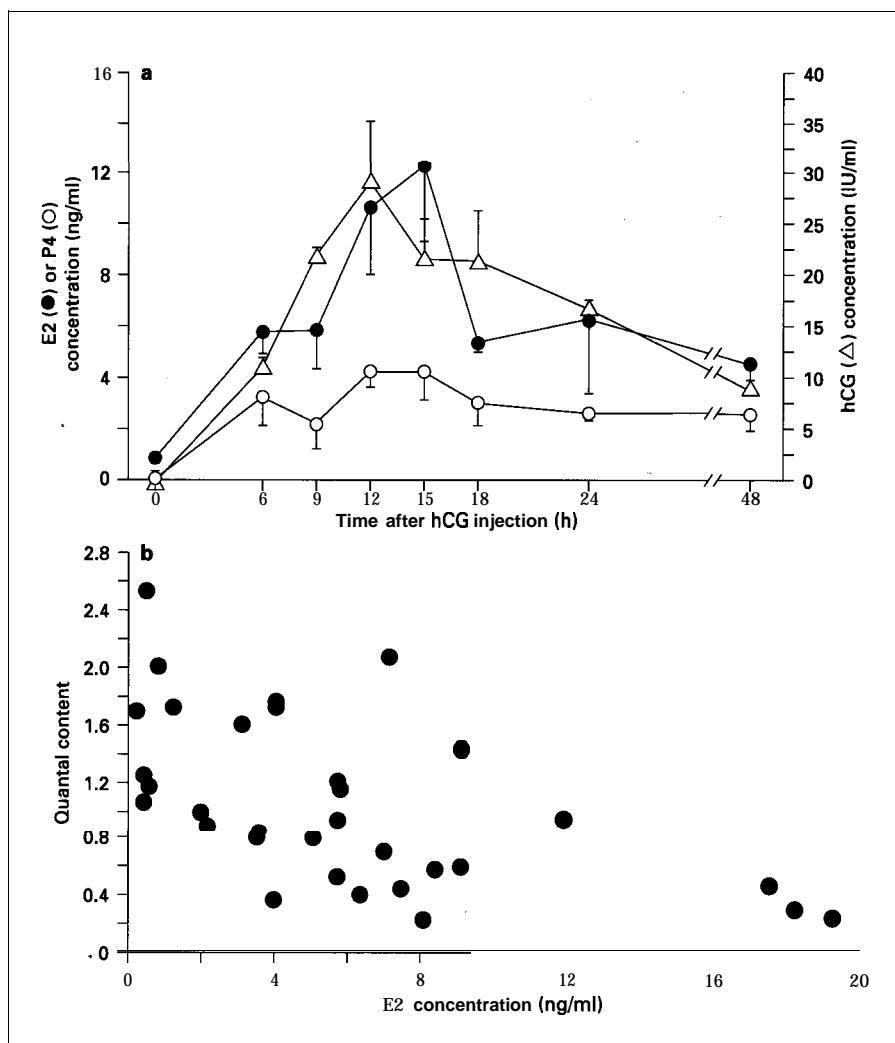
Progesterone (P4) levels also increase following hCG injection (fig. 3a). The average serum P4 level in uninjected females is 200 ± 52 (9) pg/ml (0.636 ± 0.165 nM). Overall, hCG injections induce significant increases in P4 levels ($p = 0.0002$); by 6 h after injection, P4 levels have increased significantly [$3,299 \pm 1,083$ (4) pg/ml, $p < 0.01$]. Maximum levels of P4 are obtained 12 h after injection [$4,325 \pm 637$ (6) pg/ml, $p < 0.011$]. Progesterone levels then decrease somewhat but are elevated over control values for at least 2 days after injection. hCG could not be detected in the serum of control females (< 5 mIU/ml; fig. 3a). Six hours after injection, hCG levels are 11.08 ± 1.15 (4) IU/ml. hCG levels continue to rise and are maintained at high values 9–18 h postinjection [9 h: 22.20 ± 0.51 (3) IU, $p < 0.01$; 12 h: 29.27 ± 6.37 (12) IU, $p < 0.001$; 15 h: 21.72 ± 3.99 (3) IU, $p < 0.01$; 18 h: 21.44 ± 5.06 (2) IU, $p < 0.05$; one-tailed Dunn's nonparametric comparisons]. Increased E2 and P4 levels were well correlated with elevation of hCG levels [Spearman's rank correlation; E2: $r_s = 0.752$ (32), $p < 0.001$; P4: $r_s = 0.608$ (32), $p < 0.001$]. Thus, hCG acutely, within hours, stimulates secretion of estradiol and progesterone in females.

The strength of laryngeal synapses decreases as E2 concentration increases after hCG injection [$r = -0.571$ (32), $p = 0.0005$; fig. 3b]. As might be expected from the close relation among concentration changes for the three hormones, a similar correlation is found for P4 [$r = -0.497$ (32), $p = 0.0031$]. These results confirm that laryngeal synaptic strength decreases in the short term as gonadal hormone levels increase and females become sexually receptive. Taken together with vocal production, these data indicate that call type is related to hormone titer; ticking females have low circulating levels while rapping females have high circulating levels.

Estrogen Is Responsible for the hCG-Induced Decrease in Laryngeal Synaptic Strength

To determine which hormone – hCG, estrogen or progesterone – was actually responsible for changes in synaptic strength, females ovariectomized for 10 days were

Fig. 3. The relation between hormone levels and synaptic strength after hCG injection. a Changes in estradiol, progesterone and hCG levels after hCG injection. E2 levels (filled circles) increase at 6 h and reach significantly higher levels at 12 h ($p < 0.01$) and 15 h ($p < 0.01$) after hCG injection. Progesterone (P4) levels (open circles) are significantly higher at 6 h ($p < 0.01$), 9 h ($p < 0.05$), 12 h ($p < 0.01$), 15 h ($p < 0.01$), 18 h ($p < 0.05$) and 48 h ($p < 0.05$) after hCG injection. hCG concentrations (triangles) are significantly higher than controls at 9 h ($p < 0.01$) and are maintained until 18 h after injection (12 h: $p < 0.001$; 15 h: $p < 0.01$; 18 h: $p < 0.05$). b The relation between estradiol concentration and quantal content in hCG-injected animals. A significant negative correlation was found between estradiol concentration and quantal content ($r = -0.571$, $p = 0.0005$, $n = 32$). Similar correlations with quantal content were also found for progesterone levels ($r = -0.497$, $p = 0.003$) and hCG concentrations ($r_s = -0.691$, $p < 0.001$).



injected with one of the three hormones and laryngeal synaptic strength examined 12 h postinjection (fig. 4). These intervals were chosen because laryngeal synapses are still strong while endogenous gonadal hormone levels are low 10 days after ovariectomy (fig. 6) and the maximal decrease in synaptic strength is observed 12 h after hCG injection in intact females. hCG injection does not affect synaptic strength in ovariectomized females (fig. 4a). Mean quantal contents are the same with [1.11 ± 0.20 (6)] and without [1.22 ± 0.26 (5)] hCG injection ($p = 0.735$). Acute hCG-induced decreases in laryngeal synaptic strength thus require the presence of the ovary.

Ovariectomized females have much lower estrogen levels [29.0 ± 12.0 (5) pg/ml] than intact females (901 ± 237 pg/ml; fig. 3a). hCG injection does not cause a significant increase in E2 levels in ovariectomized females [124 ± 110 (6) pg/ml, $p = 0.4571$]. Ovariectomy, which pre-

vents increases in gonadal steroid secretion after hCG injection, thus also abolishes the acute effect of hCG on synaptic strength.

Estrogen implants in ovariectomized females significantly lower quantal content; mean quantal content is 0.78 ± 0.13 (7) in E2-treated females and 1.44 ± 0.30 (7) in untreated females ($p = 0.002$; fig. 4b). Progesterone implants, in contrast, do not change laryngeal quantal content values [ovariectomized: 1.54 ± 0.23 (6), ovariectomized + P4: 1.26 ± 0.17 (6), $p = 0.3431$]. Serum E2 concentrations in implanted females are $16,414 \pm 3,280$ (7) ng/ml, levels similar to those of intact females 12-15 h after hCG injection (12 h: $10,735 \pm 6,482$ pg/ml; 15 h: $12,333 \pm 5,047$ pg/ml). We conclude that E2 is responsible for hCG-induced acute decreases in synaptic strength.

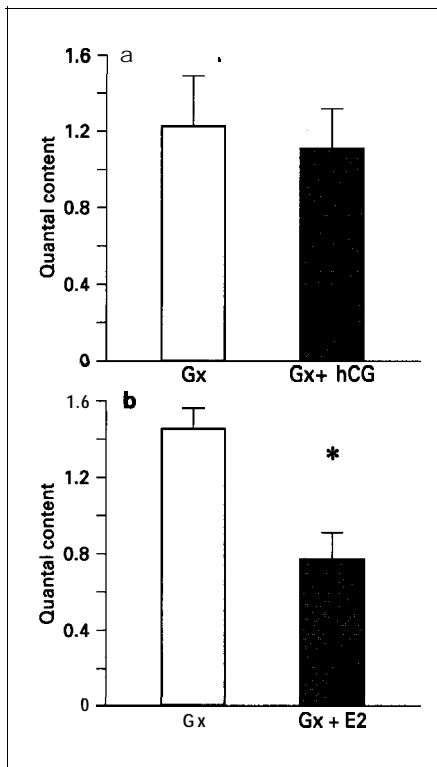


Fig. 4. Estrogen alone mimics the acute synaptic suppression of hCG-injected females. a Laryngeal synaptic strength in ovariectomized females with and without hCG injection. The two groups of animals have similarly strong laryngeal synapses. hCG injection did not suppress the strength of laryngeal synapses of ovariectomized females. Gx = Gonadectomized females; Gx+hCG = gonadectomized females with hCG injection. b Administration of estrogen causes a decrease in synaptic strength in gonadectomized females. Ovariectomized females implanted with estrogen have significantly lower quantal contents than unimplanted females (* $p < 0.005$). Gx+E2 = gonadectomized females with E2 implantation.

Long-Term Estrogen Manipulation Is Required to Increase Synaptic Strength in Juveniles and Decrease Synaptic Strength in Adult Females

That short-term decreases in laryngeal synaptic strength accompany acute increases in circulating estrogen is surprising. Previous studies indicated that the ovary is necessary for establishing and maintaining strong laryngeal synapses in adult females [6] and estrogen treatment for 2 months induces strong synapses in juveniles [5]. In all of these studies, long treatment lengths (months) were used. Since short estrogen exposures produce the opposite effect, we re-examined here the minimum period required to produce synaptic strengthening in juveniles and synapse weakening in ovariectomized adults. Quantal content values were obtained from gonadectomized

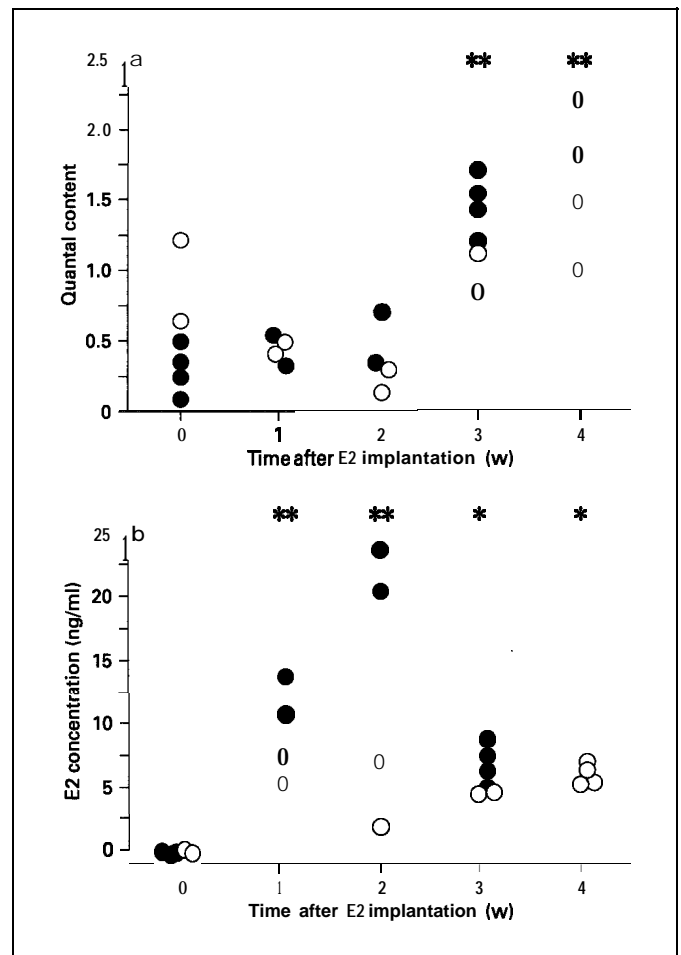


Fig. 5. Quantal content and estradiol concentration in estrogen-treated juveniles. a Changes in quantal content after E2 implantation. Results shown are from two different sets of experiments using different dosages of E2; the darker circles represent the set of experiments with higher dosages. The different dosages produce similar effects on synaptic strength. Quantal contents increase significantly from 3 weeks after implantation (** $p < 0.01$). b Changes in estradiol levels after E2 implantation. E2 levels increase significantly by 1 week after implantation (** $p < 0.01$) and are maintained at 2 weeks (** $p < 0.01$). Estrogen levels then decrease at 3 weeks but are still significantly higher than controls (* $p < 0.05$). Note the delay in the increase in quantal content when compared to the increase in E2 levels.

juveniles of both sexes at weekly intervals after treatment with E2 (fig. 5). The results presented are from two independent sets of experiments with different E2 implant dosages (supraphysiological and physiological). Effects on synaptic strength of the two dosages could not be distinguished and the data have been pooled for analysis.

Laryngeal synapses of untreated juveniles have characteristically low quantal content values [0.53 ± 0.16 (6)]

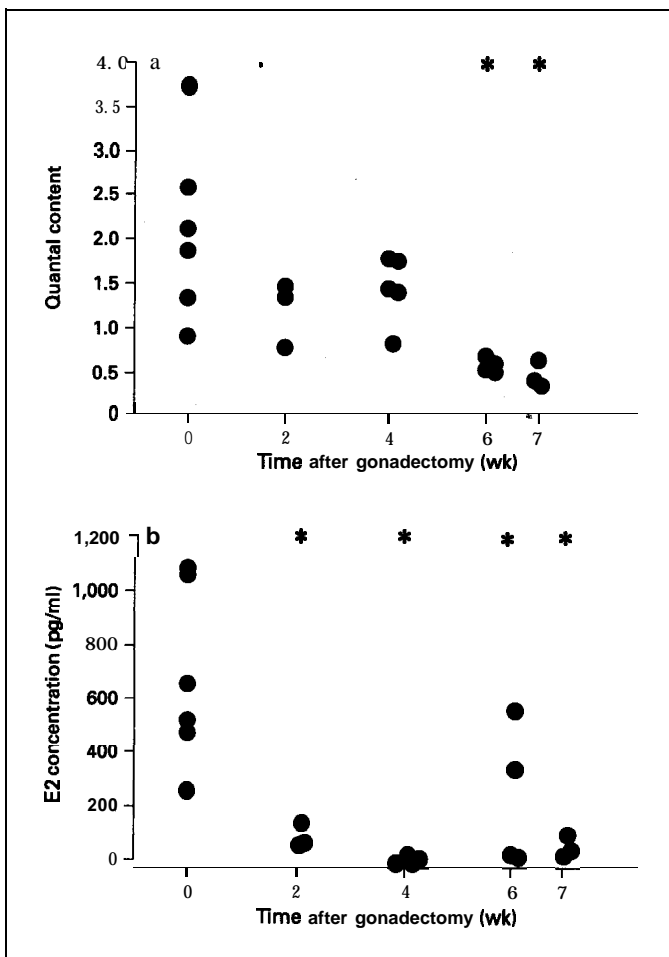


Fig. 6. Quantal content and estrogen level in ovariectomized adult females. **a** Quantal contents of ovariectomized adult females. Quantal content is significantly decreased from 6 weeks (* $p < 0.01$) after ovariectomy. **b** Serum estradiol levels in gonadectomized females. E2 levels are significantly decreased (* $p < 0.01$) 2 weeks after ovariectomy; lower levels are then maintained. Note the delay between decreases in E2 level and synaptic strength.

similar to those obtained in the previous study [5]. After 3 weeks of estrogen treatment, laryngeal synaptic strength is significantly higher than control values [fig. 5a: 1.33 ± 0.13 (6); $p < 0.01$]; these values are comparable to those of adult females (1.49 ± 0.18 ; fig. 1). Quantal content values following 1 week [0.47 ± 0.05 (4)] and 2 weeks [0.39 ± 0.12 (4)] of E2 treatment show no increase over control values. These data indicate that more than 2 weeks of estrogen exposure is required for synaptic strengthening in juveniles.

The lag between E2 implantation and increased synaptic strength was not due to a lag in circulating E2 levels (fig. 5b). While juveniles without E2 implants have very

low serum estradiol levels [2.13 ± 14.10 (6) pg/ml], implantation for 1 week produces a significant increase in E2 levels over control values [$9,443 \pm 1,883$ (4) pg/ml , $p < 0.01$, one-tailed; a one-tailed test was used since only increases in E2 levels were expected]. Two weeks after implantation, the highest E2 levels are achieved [$13,442 \pm 5,236$ (4) pg/ml , $p < 0.011$]. At 3 weeks, E2 levels decrease somewhat but are still significantly higher than controls [$6,170 \pm 724$ (6) pg/ml , $p < 0.051$]. The 2-week (minimum) lag time between a rise in circulating E2 and increases in laryngeal synaptic strength supports the hypothesis that long duration estrogen exposure is required.

Ovariectomy of adult females was known to cause a decrease in synaptic strength after 2 months [6]. We determined the minimum time required for synapse weakening by measuring quantal content bi-weekly after ovariectomy until a significant decrease in laryngeal synaptic strength was observed (fig. 6). At 2 or 4 weeks after ovariectomy, quantal content values do not differ significantly from controls [control = 2.14 ± 0.40 (6); 2 weeks = 1.26 ± 0.22 (3); 4 weeks = 1.50 ± 0.17 (5)]. Beginning at 6 weeks after ovariectomy, synapses are significantly weaker than control values [6 weeks: 0.65 ± 0.04 (4), $p < 0.01$; 7 weeks: 0.53 ± 0.08 (3), $p < 0.011$]. Thus, the effects of estrogen removal on synaptic strength are also long-term. The time course for estrogen-induced synaptic strengthening is shorter than the time course for ovariectomy-induced synapse weakening; this difference may be due either to age-related differences, juvenile versus adult, or to the effect itself, strengthening versus weakening.

The average E2 level in control females is 684 ± 135 (6) pg/ml ; this value decreases significantly to 87 ± 26 (3) pg/ml 2 weeks after ovariectomy ($p < 0.01$); low values were maintained subsequently (fig. 6b). The 2-week gap between lower E2 levels and decreased synaptic strengths again supports the idea that estrogen-induced increases in synaptic strength are long-term effects.

Females Tick with Either Strong or Weakened Laryngeal Synapses: Females Rap with Weakened Laryngeal Synapses

To compare vocal behavior with quantal content values over a wide range of synaptic strength, we compared vocalizations produced by intact females at various times after hCG injection as well as ovariectomized females (fig. 7). Larynges were dissected for quantal content determination immediately after the behavior test. Ticking is produced by intact, uninjected and by gonadectomized females. The quantal content values for these females range from 0.41 to 2.02. Quantal content values obtained

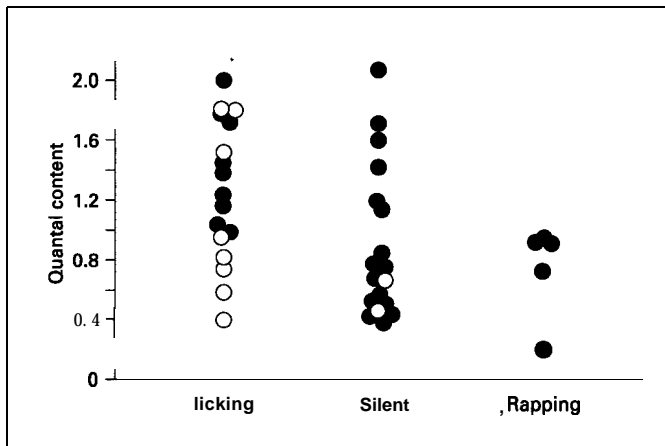


Fig. 7. Laryngeal synaptic strength and vocal behavior. Mean quantal contents of females from hCG injection and ovariectomy experiments were grouped together according to their vocal behavior. The darker circles represent animals from the hCG injection experiment (fig. 1, 2) while the lighter circles represent animals from the gonadectomy experiment (fig. 6). Females can tick or be silent with either weak or strong laryngeal synapses. However, rapping is typically observed only in females with weak laryngeal synapses.

from females that were silent range from 0.40 to 2.08; two ovariectomized females were silent when clasped by males. Thus, ticking or silence can be produced by larynges with either strong or weakened synapses. All females that rapped had low quantal content synapses. Since the only known treatment that induces rapping – gonadotropin – also weakens synapses, we have not yet been able to determine whether females with strong synapses can rap. Clearly, however, rapping does not require a strong laryngeal synapse.

Discussion

Two opposing actions of estrogen on the laryngeal synapse are apparent: a short-term depression, and a long-term enhancement of strength. Between 12 and 15 h after exposure to elevated levels of estrogen, the strength of the laryngeal synapse is depressed. Decreased quantal contents can be produced in intact females by injection of gonadotropin and in ovariectomized females by treatment with estrogen but not by hCG or progesterone treatment. Since hCG injection in intact females produces increases in circulating estrogen which parallel, but are negatively correlated with, synaptic strength, and since ovariectomy blocks the effects of hCG, we conclude that acute (hours) exposure to estrogen from the ovary tempo-

rally suppresses laryngeal synaptic strength. In contrast, long-term (>2 weeks) exposure to circulating estrogen in juveniles results in an increase in laryngeal synaptic strength. Long-term (>4 weeks) removal of estrogen by ovariectomy weakens adult female synapses. We conclude that the maintenance of strong laryngeal synapses in adult females requires long-term exposure to high levels of ovarian estrogen.

Estrogen is known to exert opposing effects on targets. For example, low doses of estradiol stimulate prostate development in male mice while high doses inhibit development [13]. With respect to neural targets, estrogen has both negative and positive feedback effects on LH and FSH secretion [14, 15]; the opposing effects depend upon the level of estrogen. However, at *Xenopus* laryngeal synapses, the opposing effects are due to duration of treatment rather than different doses. The effects of physiological and supra-physiological levels of estrogen in juveniles are identical in terms of synaptic strengthening. Provided that exposure was sufficiently long, juvenile synapses could be strengthened by estrogen levels that acutely depress synaptic strength in adults.

The mechanism of action of estrogen has been examined in a variety of excitable tissues including the nervous system, the smooth muscle of blood vessels and the uterine myometrium as well as the heart. Estrogen acts via two signalling pathways: binding to the extracellular face of cell membrane proteins and binding to the intracellular estrogen receptor, a member of a large family of ligand-activated transcription factors. Ligand-activated nuclear receptors induce alterations in gene expression via binding to specific control elements of hormone-responsive genes; by default, the membrane effects of steroids are termed 'nongenomic'. A good example of a nongenomic effect is the rapid relaxation of vascular smooth muscle in response to estrogen, an effect mediated, at least in part, by estrogen binding to the beta subunit of a potassium channel (a protein that modulates calcium sensitivity) [16]. The nongenomic effects of estrogen are generally apparent on the time scale of seconds while genomic effects do not appear until after tens of minutes. However, some very rapid effects of estrogen actually are mediated via the nuclear receptor. Estrogen stimulation of endothelial nitric oxide synthase (a vasodilator) is mediated by the nuclear receptor acting to phosphorylate a calcium-dependent, MAP kinase signalling pathway (a novel 'nongenomic' pathway) [17]. In the central nervous system, estrogen has both electrophysiological and morphological effects; the latter are generally slow, mediated via genomic actions and can involve substantial synaptic remodelling

[18]. In the system examined here, both the acute effects of estrogen and the long-term effects are relatively slow (hours to weeks) suggesting that both probably require genomic actions.

What are the cellular targets of estrogen action? Both the decrease and the increase in synaptic strength appear due to changes in neurotransmitter release from the terminals of laryngeal motor neurons. In adult males and females, a very robust difference in quantal content is present without any discernible difference in the postsynaptic response (as assessed by mEPSP amplitudes) [4]. We show here that the decrease in quantal content produced by acute exposure to estrogen is also not accompanied by a decrease in quantal size. These observations suggest an action of estrogen on the laryngeal motor neuron. However, this cell does not appear to express estrogen or progesterone receptors either in *Xenopus* or in mammals [19, 20] though androgen receptors are expressed at high levels [21, 22]. Thus genomically mediated effects on the laryngeal motor neuron are probably transsynaptic in nature, either anterograde influences from neurons in ventral diencephalon, thalamus and auditory midbrain [22, 23] or retrograde influences from laryngeal muscle which does express estrogen receptor mRNA [Wu, Tobias, Kelley, unpubl. observations].

Estrogen effects on synaptic strength are presynaptic and thus reflect changes in transmitter release. These effects include those producing sex differences in synaptic strength as well as those that accompany changes in sexual state in females. Transmitter release at the active zone is affected both by calcium influx via ion channels and by sensitivity to calcium required for release mediated by calcium-dependent synaptic proteins. The active zone is characterized by a dense array of both voltage-activated calcium channels, required for calcium influx, and calcium-activated potassium channels, which repolarize the terminal [24]. In mammalian muscle, estrogen effects on potassium currents are prominent. For example, estrogen rapidly inhibits potassium currents in heart cells thus increasing action potential duration [25]; in gonadotropes estrogen, in contrast, acts more slowly to increase potassium currents [26]. Potassium channels present in high concentrations at active zones are thus candidate downstream estrogen targets at the laryngeal synapse; calcium channels are another candidate [24]. Calcium-binding proteins of the presynaptic terminal such as synaptotagmin and frequenin are believed to regulate vesicle release and thus synaptic strength; their characteristics thus help to determine the calcium sensitivity for release. Synaptotagmins act as calcium sensors for vesicle exocytosis;

these proteins bind to the phospholipid bilayer in response to calcium influx. A number of synaptotagmin isoforms have been cloned; the relation between phospholipid binding and calcium concentration varies for different isoforms [27]. In *Xenopus*, frequenin overexpression enhances synaptic efficacy [28]. Possible mechanisms include modulation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger [29] and/or phosphoinositide signalling [30]. Thus, calcium sensitivity modulation (male versus female, receptive versus unreceptive, short- versus long-term estrogen exposure) might be achieved via differential expression of synaptotagmin isoforms or regulation of frequenin levels.

Synaptic remodelling is an additional mechanism for alterations in release following long-term estrogen exposures. In the hippocampus, synaptic remodelling accompanies changing estradiol levels during the estrous cycle [31]. Elevated levels of estradiol are associated with a higher density of dendritic spine synapses on CA1 pyramidal cells. Possible remodelling of the laryngeal synapse in response to long-term exposure to estradiol could also contribute to increases in synaptic strength by, for example, increases in numbers of synaptic active zones. Previous studies have revealed no sex differences in active zone length or in the density of channels associated with the active zone [4].

The initial observation of sexually differentiated laryngeal synapses raised the possibility that this characteristic contributes to sex specific vocal features. For example, we suggested that females, with fewer laryngeal fibers, might require strong synapses to insure that enough muscle fibers contract to produce the clicks in ticking [6]. Strong synapses were predicted to be particularly important in producing the louder, faster clicks of rapping [7]. The association between synaptic strength and vocal output seen here is more complex than this simple scenario. Gonadotropin treatment of intact females is accompanied by rapping and weakening of synaptic strength. Females can tick with either strong or weakened synapses; ovariectomized females, with very low quantal contents, as well as uninjected, intact females, with high quantal contents, both tick.

Quantal content values for animals that rapped are within the range for females that ticked. The only reliable relation between vocalization and synaptic strength is the association of rapping with weakened synapses. We cannot determine currently whether females with stronger synapses can rap because our only means of inducing rapping, hCG injection, also reduces quantal content. However, it is clear that strong synapses are not required for any female vocal behavior.

What is meant by 'weakened' synapses? To measure quantal content, the synapse must be studied under non-physiological conditions that reduce transmitter release and muscle contraction. Under these artificial conditions, the laryngeal synapse is weaker in gonadotropin-injected females and stronger in uninjected females. However, since both injected and uninjected females call, we assume that, under physiological conditions, the synapse in injected females is strong enough to produce muscle action potentials and actual clicks. In fact, one role for strong synapses in females might be to maintain transmission despite the weakening effects of gonadotropin secretion required for ovulation. In this scenario the strong synapses of females maintain their ability to display the

full range of vocal signals used in advertising sexual state to males. Alternatively, the primary target for estrogen action on the vocal system may be central rather than peripheral. For example, estrogen may affect vocalization by acting on its neural targets [19]; effects on laryngeal synapses described here would then be incidental to the major, central activity of this steroid hormone.

Acknowledgments

This work was supported by National Institutes of Health Grant NS 23864 (D.B.K.) and an NIH training grant NS 07062 (K.H.W.). The authors thank Lieqi Liu for performing radioimmunoassay.

References

- Kelley DB, Tobias ML: Vocal communication in *Xenopus laevis*; in Hauser M, Konishi M (eds): Neural Mechanisms of Communication. New York, MIT Press, 1999, pp 9-35.
- Tobias ML, Kelley DB: Vocalizations by a sexually dimorphic isolated larynx: Peripheral constraints on behavioral expression. *J Neurosci* 1987;7:3191-3197.
- Tobias ML, Kelley DB: Electrophysiology and dye-coupling are sexually dimorphic characteristics of individual laryngeal muscle fibers in *Xenopus laevis*. *J Neurosci* 1988;8:2422-2429.
- 4 Tobias ML, Kelley DB, Ellisman M: A sex difference in synaptic efficacy at the laryngeal neuromuscular junction of *Xenopus laevis*. *J Neurosci* 1995; 15:1660-1668.
- Tobias ML, Kelley DB: Sexual differentiation and hormonal regulation of the laryngeal synapse in *Xenopus laevis*. *J Neurobiol* 1995;28: 515-526.
- Tobias ML, Tomasson J, Kelley DB: Attaining and maintaining strong vocal synapses in female *Xenopus laevis*. *J Neurobiol* 1998;37: 441-448.
- Tobias ML, Viswanathan SS, Kelley DB: Rapping, a female receptive call, initiates male-female duets in the South African clawed frog. *Proc Natl Acad Sci USA* 1998;95: 1870-1875.
- 8 Kelley DB: Female sex behaviors in the South African clawed frog, *Xenopus laevis*: Gonadotropin-releasing, gonadotropic, and steroid hormones. *Horm Behav* 1982; 16:158-174.
- 9 Del Castillo J, Katz B: Quantal components of the end-plate potential. *J Physiol* 1954; 124: 560-573.
- 10 Westley B, Knowland J: An estrogen receptor from *Xenopus laevis* liver possibly connected with vitellogenin synthesis. *Cell* 1978;15:367-374.
- 11 Zar JH: Biostatistical Analysis, 2nd ed. Englewood Cliffs, Prentice-Hall, 1984.
- 12 Liao D, Jones A, Malinow R: Direct measurement of quantal changes underlying long-term potentiation in CA 1 hippocampus. *Neuron* 1992;9:1089-1097.
- 13 vom Saal FS, Timms BG, Montano MM, Palanza P, Thayer KA, Nagel SC, Dhar MD, Ganjam VK, Parmigiani S, Welshons WV: Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. *Proc Natl Acad Sci USA* 1997;94:2056-2061.
- 14 Mahesh VB, Brann DW: Steroid control of hypothalamic function in reproduction; in Stone TW (ed): CNS Neurotransmitters and Neuromodulation: Neuroactive Steroids. Boca Raton, CRC Press, 1996, pp 177-209.
- 15 Fink G: Oestrogen and progesterone interactions in the control of gonadotropin and prolactin secretion. *J Steroid Biochem* 1988;30:169-178.
- 16 Valverde MA, Rojas P, Amigo J, Cosmelli D, Orio P, Bahamonde MI, Mann GE, Vergara C, Latorre R: Acute activation of Maxi-K channels (hSlo) by estradiol binding to the beta subunit. *Science* 1999;285:1929-1931.
- 17 Shaul PW: Novel role of estrogen receptors in vascular endothelium. *Semin Perinatol* 2000; 24:70-74.
- 18 Woolley CS: Estrogen-mediated structural and functional synaptic plasticity in the female rat hippocampus. *Horm Behav* 1998;34: 140-148.
- 19 Morrell JI, Kelley DB, Pfaff DW: Autoradiographic localization of hormone-concentrating cells in the brain of an amphibian, *Xenopus laevis*. II. Estradiol. *J Comp Neurol* 1975; 164:63-77.
- 20 Simerly RB: Distribution and regulation of steroid hormone receptor gene expression in the central nervous system. *Adv Neurol* 1993;59: 207-226.
- 21 Kelley DB: Locations of androgen-concentrating cells in the brain of *Xenopus laevis*: Autoradiography with ³H-dihydrotestosterone. *J Comp Neurol* 1981;199:221-231.
- 22 Kelley DB, Morrell JI, Pfaff DW: Autoradiographic localization of hormone-concentrating cells in the brain of an amphibian, *Xenopus laevis*. I. Testosterone. *J Comp Neurol* 1975; 164: 47-59.
- 23 Kelley DB: Auditory and vocal nuclei in the frog brain concentrate sex hormones. *Science* 1980;207:553-555.
- 24 Robitaille R, Garcia ML, Kaczowski GJ, Charlton MP: Functional colocalization of calcium and calcium-gated potassium channels in control of transmitter release. *Neuron* 1993; 11: 645-655.
- 25 Tanabe S, Hata T, Hiraoka M: Effects of estrogen on action potential and membrane currents in guinea pig ventricular myocytes. *Am J Physiol* 1999;277:H826-833.
- 26 Cowley MA, Chen C, Clarke IJ: Estrogen transiently increases delayed rectifier, voltage-dependent potassium currents in ovine gonadotropes. *Neuroendocrinology* 1999;69:254-260.
- 27 Marqueze B, Berton F, Seagar M: Synaptotagmins in membrane traffic: which vesicles do the tagmins tag? *Biochimie* 2000;82:409-420.
- 28 Olafsson P, Wang T, Lu B: Molecular cloning and functional characterization of the *Xenopus* Ca²⁺-binding protein frequenin. *Proc Natl Acad Sci USA* 1995;92:8001-8005.
- 29 Pongs O, Lindemeier J, Zhu XR, Theil T, Engelkamp D, Krah-Jentgens I, Lambrecht HG, Koch KW, Schwemer J, Rivosecchi R, Mallart A, Galceran J, Canal I, Barbas JA, Ferrús A: Frequenin - A novel calcium-binding protein that modulates synaptic efficacy in the *Drosophila* nervous system. *Neuron* 1993; 11: 15-28.
- 30 Hendricks KB, Wang BQ, Schnieders EA, Thorner J: Yeast homologue of neuronal frequenin is a regulator of phosphatidylinositol-4-OH kinase. *Nat Cell Biol* 1999;1:234-241.
- 31 Woolley CS: Effects of estradiol on hippocampal circuitry. *Novartis Found Symp* 2000;230: 173-180.