

Attaining and Maintaining Strong Vocal Synapses in Female *Xenopus laevis*

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ABSTRACT: Synaptic efficacy at the laryngeal neuromuscular synapse differs markedly in adult male and female *Xenopus laevis*. Here, we examined the relation between circulating estrogen and synapse strength in developing and adult female frogs. Circulating estrogen levels in males and females during juvenile and adult stages were measured using radioimmunoassays. Synaptic strength was determined by quantal analysis in isolated female larynges. In males, estrogen levels are low (<40 pg/mL) throughout development. In females, estrogen levels are similar to those in males until 9 months after metamorphosis is complete and then increase throughout development. Female laryngeal synapses have low quantal contents until 24 months; quantal content increases significantly between 24 and 26 months, and high quantal contents are maintained thereafter. Measures of reproductive maturation, ovary,

and oviduct weights, are strongly and positively correlated with estrogen level in 16- to 26-month females, while oocyte maturation is age dependent. Estrogen level and quantal content are not well correlated in these females. Ovariectomy at 24 months prevents the expected increase in quantal content and ovariectomy at 28 months results in a decrease in quantal content. Thus, the sex difference in efficacy of the laryngeal synapse develops under the influence of the ovary and requires the ovary for maintenance of strong synapses in adulthood. While the influence of the ovary is most likely due to estrogen secretion, the pattern of estrogen secretion required for maturation of the synapse in females is not known. © 1998 John Wiley & Sons, Inc. *J Neurobiol* 37: 441–448, 1998

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Male and female *Xenopus laevis* frogs use distinct vocal behaviors to convey information about receptive state and location to potential mates (Tobias et al., 1998; Kelley, 1996). Vocalizations are produced by contraction of laryngeal muscles in response to activity of laryngeal motor neurons. Sex differences in the strength of the synapse between the laryngeal motor neuron and muscle fiber contribute to sex differences in song. The laryngeal synapse is stronger in adult females than in males owing to greater transmitter release from the motor terminal (Tobias et al., 1995). The weak laryngeal synapse of males permits amplitude modulation of sound, a prominent feature of

male courtship songs (Tobias and Kelley, 1987; Tobias et al., 1998), while the stronger laryngeal synapse of females guarantees sound production despite a smaller number of muscle fibers and motor neurons (Kelley and Dennison, 1990; Marin et al., 1990).

These sex differences can be traced to male- and female-specific developmental programs. In sexually immature juveniles of both sexes, synaptic strength is weak or male-like and can be increased by exogenous estrogen treatment (Tobias and Kelley, 1995). These observations suggest that adult sex differences are the result of differences in the secretion of gonadal steroids in males and females, and specifically that estrogen secretion is responsible for the strong synapses of the adult female.

The goal of this study was to follow estrogen secretion and synapse strength during development and adult-

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hood by obtaining estrogen level and laryngeal quantal content from the same individual. In addition, we determined whether the ovaries are required for the development and maintenance of strong synapses.

MATERIALS AND METHODS

Animals

Postmetamorphic juveniles and adults were purchased from Nasco (Ft. Atkinson, WI) and maintained in polycarbonate aquaria; water was carbon filtered and treated with Novaqua to remove heavy metals. Frogs were fed frog brittle (Nasco) three times per week and kept on a 12:12 h light/dark cycle. Quantal content and estrogen level measurements were obtained from November to March. Ages reported here refer to the number of months after the completion of metamorphosis.

Estrogen Measurements

Frogs were anesthetized in MS222 (0.1%; *m*-amino benzoate, methane sulfonic acid; Aldrich Co.). The pericardium was removed and blood was collected directly from the ventricle. Samples were placed on ice for 15 min and centrifuged, and the plasma was then collected and stored at -20°C until assay. Estrogen was assayed using the Coat-A-Count Estradiol radioimmunoassay system (Diagnostic Products Corp.); data provided by the manufacturer indicate that this antiserum is specific for 17β -estradiol with 10% cross-reactivity to estrone and 1.8% cross-reactivity to estrone- β -*D*-glucuronide. Because of cross-reactivity of the antiserum to other estrogens, we refer to estrogen level rather than estradiol level in this article. The sensitivity of the assay (two standard deviations below maximal binding) is 8 pg/mL. Assays were carried out as specified by the manufacturer; for each assay, standard curves from serial dilutions were prepared and zeroed against blanks, and extraction efficiencies were estimated using radiolabeled estradiol. Each data point represents two samples from one animal except for the 6-month group, which included pooled data from two or three animals.

Quantal Content

After collecting blood samples, the larynx was removed and pinned dorsal side up in a petri dish superfused with normal frog saline (116 mM NaCl, 2 mM KCl, 2.5 mM CaCl_2 , 3.0 mM MgCl_2 , 27.7 mM dextrose, 4 mM Hepes buffer). Connective, fat, and cartilaginous tissues were removed to expose the nerve and musculature of the larynx. The laryngeal nerve was drawn into a suction electrode and used to stimulate the muscle (Grass S8800). The normal saline was then replaced with low-calcium/high-magnesium saline (105 mM NaCl, 2 mM KCl, 0.5 mM CaCl_2 , 5.0 mM MgCl_2 , 27.7 mM dextrose, 4 mM Hepes buffer) and allowed to incubate until visible muscle twitches in response to nerve stimulation were blocked (typically 2–3 h). Stimulus inten-

sity was gradually increased until visible twitches were observed in normal saline. This stimulus intensity was maintained for subsequent intracellular recordings in low-calcium/high-magnesium saline. The effective stimulus intensity varied between animals because of the tightness of fit between the suction electrode and the nerve; stimulus intensities were within 1–15 mA for all animals. Intracellular glass microelectrodes (30–60 M Ω) were used to impale laryngeal muscle fibers; only fibers with a resting potential of $\geq -70\text{mV}$ were studied. The number of failures in response to 150 trials of nerve stimulation was determined and used to calculate quantal content (\ln [number of trials/number of failures]) (del Castillo and Katz, 1954) (see Fig. 3). For each animal, mean quantal content values were determined from three muscle fibers.

Ovariectomy

Animals were anesthetized as above and a small incision through the skin and musculature was made to one side of the abdominal midline. Ovaries and associated fat bodies were gently retracted and severed with cautery from their attachment to the interrenal gland. The incisions were then sutured and the animal was allowed to recover in moist towels before returning to the aquarium.

Body, Ovary, and Oviduct Weights and Oocyte Maturation

All animals were weighed following anesthesia. After collecting blood and removing the larynx, the ovaries and oviducts of the females were removed and weighed. The left reproductive organs were frequently heavier than the right; the mean value was determined for each animal. Oocytes were categorized as belonging to one of six stages of maturation (Dumont, 1971) according to size and extent of pigmentation. For example, stage 6 (mature) oocytes are large (1200 μm) and have an equatorial band dividing the animal (pigmented) and vegetal (unpigmented) poles. Stage 1 oocytes (immature) are small (50–300 μm) and yolckless. Since the female ovary can contain oocytes at different stages of maturation, oocyte maturation was determined by the most mature oocyte stage present in the ovary examined with the aid of a dissecting microscope.

Statistics

The Spearman rank correlation coefficient was used to test the significance of correlations between variables. Treatment and control groups were compared using the Mann-Whitney *U* test. Means and standard deviations are given for all measurements.

RESULTS

Estrogen Levels during Development

At 6 months estrogen levels were low (<25 pg/mL) and similar in the sexes [Fig. 1(A)]. By 9 months estrogen

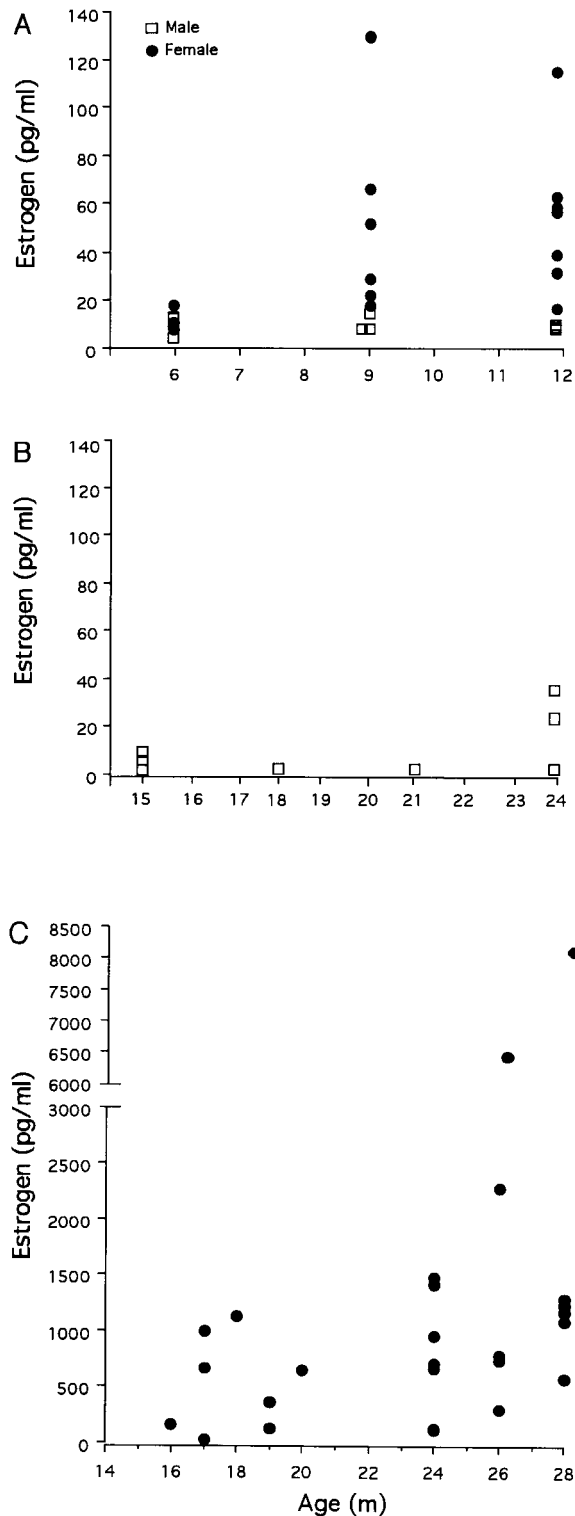


Figure 1 Estrogen level in male and female *X. laevis* during postmetamorphic development. (A) Estrogen level in juvenile (6- to 12-month) males and females. Estrogen levels are significantly higher in females by 9 months. (B) Estrogen level in older (15- to 24-month) males. (C) Estrogen level in older (16- to 28-month) females. Note change in scale.

levels were higher in females than in males (female mean = 49.3 ± 34.9 ; male mean = 11.6 ± 7.7 ; $p < .02$) and remained so throughout development. Male estrogen levels continued to be low throughout development into adulthood [Fig. 1(B)]. Female mean estrogen levels continued to increase [Fig. 1(C)]. While estrogen levels of individuals within one age group varied considerably, age and estrogen level were positively correlated ($r = 0.8$; $p < .0001$).

Estrogen Levels and Reproductive Maturation

We next determined the relation between estrogen level and reproductive maturation in females at 16–28 months (Fig. 2). For each female, reproductive maturation was assessed by determining ovary and oviduct weights as well as oocyte maturation. Estrogen level and either ovary ($r = 0.8$; $p < .0001$) or oviduct ($r = 0.7$; $p = .0006$) weights were well correlated. Mature oocytes were present in some females prior to 24 months and were present in all females from 24 months on. Rising levels of estrogen thus accompany sexual maturation in females.

Estrogen, Age, and Synapse Strength

Synapse strength and estrogen level were examined in females at 16–28 months [Fig. 3(A)]. Quantal contents were low prior to sexual maturity (<24 months = 0.6 ± 0.3) and remained low at 24 months (0.7 ± 0.3), the onset of sexual maturity. Quantal content values for laryngeal synapses of 24-month females did not differ significantly from values obtained in younger animals ($p = .7$). At 26 months quantal content values increased dramatically (2.1 ± 0.3); these greater values were maintained at 28 months (1.6 ± 0.7). Quantal content values were significantly higher in females 26 months or older compared with values from younger females ($p < .0001$). We conclude that laryngeal synapse strength in females is highly age dependent and matures at some point between 24 and 26 months.

Estrogen level and quantal content were not significantly correlated [Fig. 3(B)] ($r = 0.4$; $p = .1$). A female with a high estrogen level can have laryngeal synapses with low quantal contents, and vice versa. Thus, estrogen level alone is a poor predictor of synapse strength.

Role of the Ovary in Modulation of Synapse Strength

We next determined whether the attainment of strong laryngeal synapses depends on the presence of the

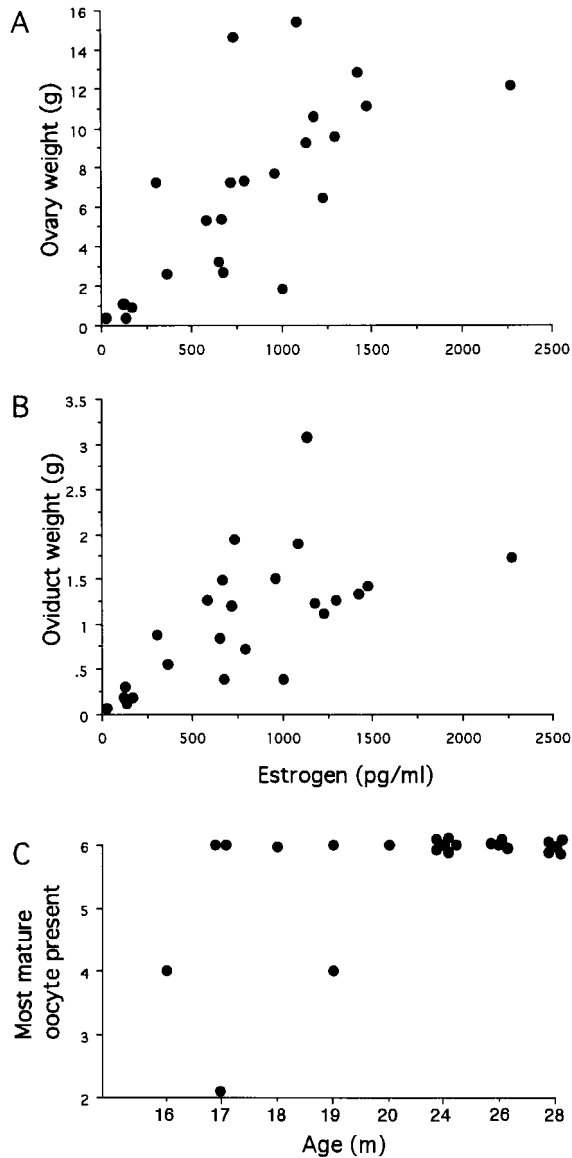


Figure 2 Estrogen levels increase with reproductive maturation. Estrogen level and ovary weight (A), oviduct weight (B), and oocyte maturation (C) are shown. All females were between 16 and 28 months. The two highest estrogen values observed at 26 and 28 months [see Fig. 1(C)] are not included but are in the correlation statistic.

ovaries. Females were ovariectomized or received a sham operation at 23 months, prior to developing strong synapses, and the quantal content of laryngeal synapses was determined at 26 months, when strong synapses were present in intact females (Fig. 4). Ovariectomy prevented the expected strengthening of the laryngeal synapse. Quantal content values for laryngeal synapses of 26-month females ovariectomized at 23 months did not differ significantly from values for gonadally intact 23-month females (23 months ovx/26 months = 1.22 ± 0.1 , 23 months

= 0.7 ± 0.7 ; $p = .2$). However, quantal content values for laryngeal synapses from these ovariectomized 26-month females were significantly less than values obtained from 26-month females that received a sham operation at 23 months (2.3 ± 0.7 ; $p = .01$). We conclude that ovariectomy blocks the rise in quantal content that normally occurs at 26 months.

We next determined whether the ovary was required to maintain strong synapses in older females. Twenty-eight-month females were ovariectomized and quantal content was determined at 34 months (Fig. 5). Quantal content values were significantly lower in ovariectomized females (0.8 ± 0.5) than in age-matched 34-month (1.6 ± 0.4) or intact 28-month (1.6 ± 0.7) controls ($p = .04$ and $.05$, respectively). Thus, the presence of the ovary is required to maintain strong synapses. Because quantal content values of laryngeal synapses remained high at 34 months, the decrease observed in the ovariectomized females was not due simply to aging.

Ovariectomy significantly reduced the level of circulating estrogen in 23-month (1126 ± 1102 pg/mL in intact 23-month, 604 ± 474 pg/mL in intact 26-month, and 18 ± 9 pg/mL in 23-month ovx/26-month females; $p = .05$ and $.02$, respectively) and 26-month females (2931 ± 2894 pg/mL in intact 26-month, 3560 ± 2002 pg/mL in intact 34-month, and 210 ± 280 pg/mL in 26-month ovx/34-month females; $p = .004$ and $.01$, respectively).

DISCUSSION

The sexually differentiated features of the adult larynx include a robust difference in synapse strength. Under physiological conditions, a single stimulus applied to the laryngeal nerve does not evoke action potentials in male muscle fibers, while muscle action potentials follow each nerve impulse in females (Tobias and Kelley, 1988). Quantal content measurements from adults reveal weaker synapses in males than females (Tobias et al., 1995). In juveniles of both sexes, laryngeal synapses are also weak (Tobias and Kelley, 1995). Thus, males maintain their weak synapses into adulthood; here, we sought to determine when during development female synapses become strong. Our results indicate that the attainment of strong synapses is a very late event in female development. For the first 6 months, quantal content values of male and female juveniles are weak (0.5 ± 0.4) (Tobias and Kelley, 1995) and resemble those of adult males (0.5 ± 0.3) (Tobias et al., 1995). Older juvenile females (16–20 months) and sexually mature young adults (24 months) maintain weak synapses; values typical of fully mature females were not achieved until 26

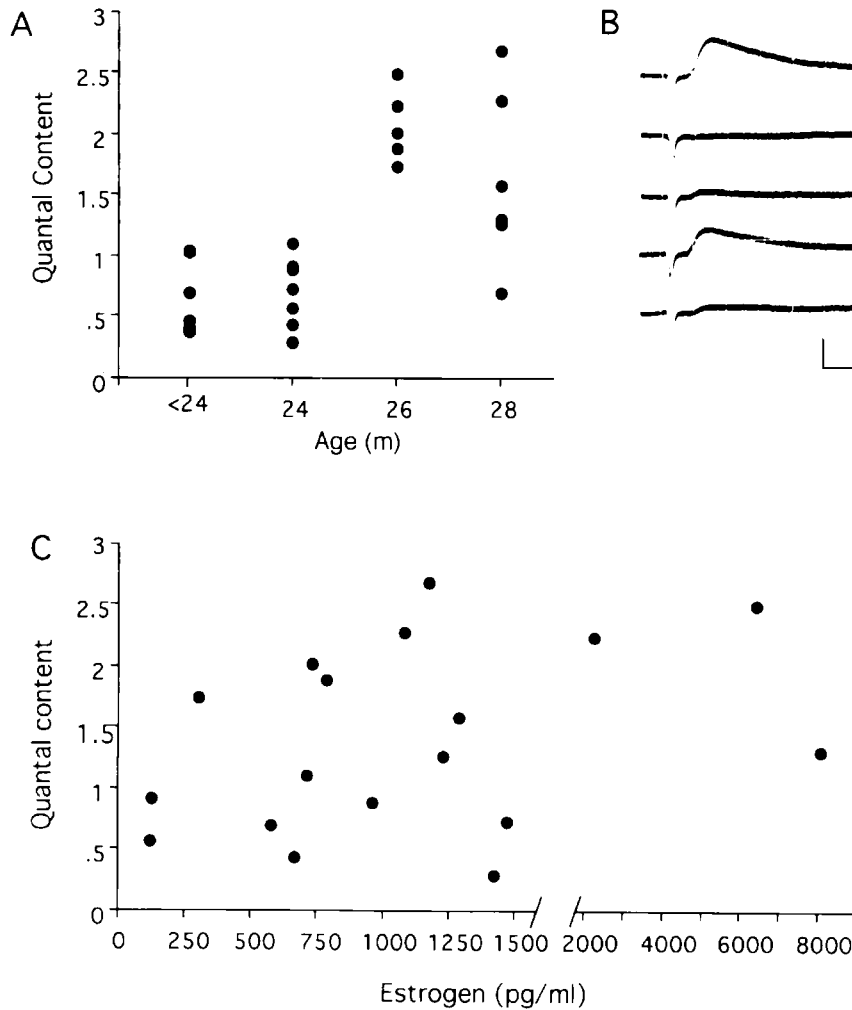


Figure 3 Quantal content is strongly age dependent but not correlated with estrogen level. (A) The relation between quantal content and age. The <24-month group represents one female each at 16, 18, and 20 months; two females at 19 months; and three females at 17 months. (B) Intracellular records from a 26-month-old female; the records depict responses to consecutive stimulus trials. This synapse produced a post synaptic potential (psp) in response to all stimulus trials except the second. Calibration bars: 10 mV, 5 ms. (C) The relation between quantal content and estrogen level. Each quantal content value represents the mean of three synapses per female.

months and were maintained until 34 months, the oldest females examined in this study.

Developing and maintaining strong synapses in females depends on the presence of the ovary. Ovariectomy at 23 months, when synapses have juvenile quantal content values, blocks the attainment of adult-typical and ovariectomy at 28 months, after maturation of quantal content, results in juvenile-like values. Thus, the ovary is required both to produce and maintain high quantal content values.

Estrogen and Synapse Strength

The sex difference in laryngeal synaptic strength is presynaptic (Tobias et al., 1995). One hypothesis for

the effect of the ovary on synaptic maturation is that secretion of estrogenic steroids influences neurotransmitter release. This hypothesis arose from the observation that exogenous estrogen increases quantal content values at laryngeal synapses in juveniles of both sexes (Tobias and Kelley, 1995). If estrogen controls synapse strength, we might expect levels of endogenous estrogen to rise at or just before the developmental stage at which synapse strengthening is first observed, 26 months. In addition, if synapse strength reflects contemporaneous circulating levels of estrogen, we might expect a tight correlation between estrogen level and quantal content obtained from the same female. Males and young females (<9 months) have very low estrogen levels and very low quantal

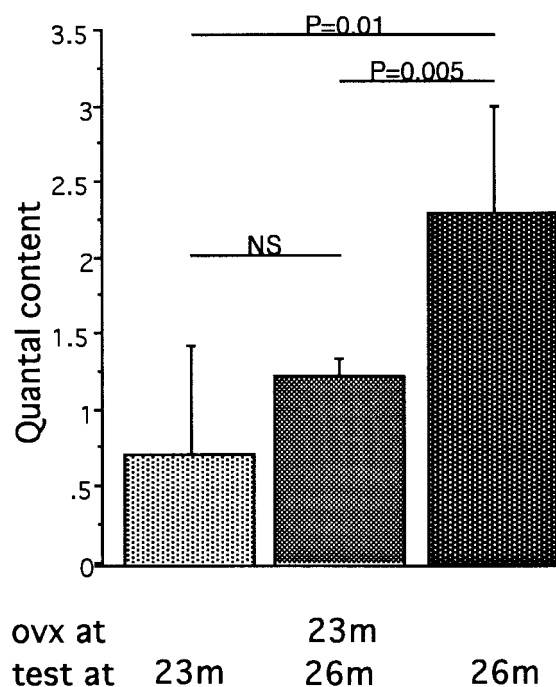


Figure 4 Ovariectomy at 23 months prevents the expected increase in quantal content. Mean quantal content (\pm SD) values are shown for untreated 23-month females ($n = 4$ animals), females ovariectomized at 23 months and examined at 26 months ($n = 6$ animals), and females that received a sham operation at 23 months and were examined at 26 months ($n = 5$ animals).

content values. While estrogen levels then start to increase in females, we did not detect evidence of an abrupt increase at or before 26 months, when quantal content increases markedly. Synapse strength can still be weak when other reproductive characteristics—for example, oocyte development—are fully mature. In females examined from 16 to 28 months, synapse strength is age dependent but is not correlated with estrogen level.

Given these observations, an alternative hypothesis is that quantal content values reflect a preceding history of estrogen exposure, a history not accurately conveyed by a single determination of circulating levels. For example, mean estrogen levels within an age group consistently increase throughout development, suggesting that older females have been exposed to more estrogen for longer periods than younger females, even if the value obtained at a single time point is low. A long-lasting or “memory” effect of exposure to estradiol has been demonstrated in the liver of male *X. laevis*; both the latency and accumulation of vitellogenin mRNA are increased following a second estrogen exposure, suggesting that the history of estrogen exposure has a stable and long lived influence on gene expression (Baker and Shapiro,

1978). Alternatively, estrogen levels might increase sharply just prior to ovulation and some period of very high levels of estrogen exposure be required for establishing or maintaining strong synapses. If estrogen secretion is cyclic, estrogen levels may vary dramatically in the same animal at different times depending on cycle duration. Although seasonal changes in estrogen level have been described in the bullfrog, *Rana catesbeiana* (Licht et al., 1983), and in the European frog, *Rana esculenta* (D’Istria et al., 1974), it is not known whether shorter cycles, within a season, exist as well. Measurements of quantal content and estrogen level in this study were obtained between November and March, a period outside the breeding season in the wild (King and Millar, 1979). The frogs examined here were laboratory reared, a factor which could influence circannual hormone cycles.

Another possibility is that the effects of estrogen are cumulative, so that even low circulating levels can strengthen synapses as long as they are present over a long time or succeed a previous higher level. In this and the preceding study (Tobias and Kelley, 1995), the duration of effective hormonal manipulations were long (6–12 weeks in juveniles and 6 months in adults); the duration and threshold levels for the

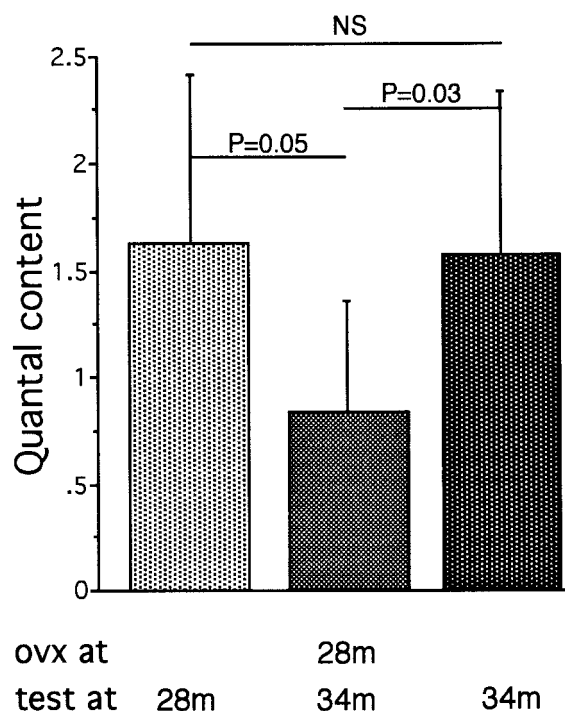


Figure 5 Ovariectomy at 28 months depresses synapse strength at female laryngeal synapses. Mean quantal content (\pm SD) are shown for untreated 28-month females ($n = 6$ animals), females ovariectomized at 28 months and examined at 34 months ($n = 6$ animals) and intact 34-month females ($n = 5$ animals).

strengthening of laryngeal synapses using exogenous estrogen remain to be determined.

Finally, it is possible that the ovary secretes factors other than estrogen which contribute to synapse strengthening; candidates include progestins and androgens. One role for progesterone is to trigger ovulation, which it does by acting directly on the follicular cells; progesterone also triggers meiosis (oocyte maturation) by acting directly on the oocytes (reviewed in Dumont, 1971). While the effects of progesterone on synapse strength in the larynx have not been examined, we found no positive correlation between progesterone levels and synaptic strength in females during postmetamorphic development (unpublished observations). Androgens sensitize the pituitary to luteinizing hormone releasing hormone (LHRH) in female bullfrogs, which should also increase estrogen levels (Licht et al., 1983; McCreary and Licht, 1984). Exogenous androgen treatment, however, does not affect synapse strength in juveniles, although the juvenile larynx expresses high levels of androgen receptor (Kelley et al., 1989; Fischer et al., 1993; Tobias and Kelley, 1995). Ovarian secretion of androgen might also provide a substrate for local aromatization to estrogen at laryngeal synapses, as has been observed in synaptosomal fractions of quail brain (Schlinger and Callard, 1989). However, given that prolonged treatment with exogenous estrogen does produce female-typical quantal content values in juveniles whose normal circulating levels are <25 pg/mL, any additional influence of the ovary apparently can be mimicked by exogenous estrogen.

Feminization and Masculinization of Laryngeal Development

Synaptic strength becomes sexually differentiated in a way that differs markedly from sexual differentiation of other cellular characteristics of the larynx. Masculinization of muscle fiber number and type, for example, relies on gonadal androgen secretion during early juvenile development. The capacity for masculinization is maintained into adulthood; however, once masculinized, cell number and type do not revert to a sexually undifferentiated form even when the hormone source is removed (reviewed in Kelley, 1996; Kelley and Tobias, 1998). For laryngeal cell number and type, the default phenotype (that exhibited in the absence of the gonads) is female; testicular secretions irreversibly transform these characteristics. For synaptic strength, on the other hand, the default phenotype is male and the fully feminized synapse does not appear until adulthood. Ovarian secretions are required both for the increase in synaptic strength that accompanied feminization and for the maintenance of

strong synapses; effects on quantal content are reversible. The behavioral effects of hormones have historically been viewed as falling into two classes: organizational or permanent effects due to hormone exposure during development and activational, or reversible effects due to fluctuations in hormone levels during adulthood (Phoenix et al., 1959; Arnold and Breedlove, 1985). The effects of estrogen on synaptic strength are activational.

Estrogens and Synaptic Strength: Mechanism and Functional Implications

The male laryngeal synapse is so weak that single action potentials do not release enough transmitter to depolarize the muscle membrane to threshold; the male synapse relies on synaptic facilitation to produce muscle action potentials (Ruel et al., 1998). Our results suggest that ovarian secretions allow females to overcome this intrinsic weakness. Estrogen has also been shown to affect synaptic efficacy in the rodent hippocampus. Two effects on pyramidal CA1 cells have been studied: an increase in spine density and an increased sensitivity to *N*-methyl-D-aspartate-mediated synaptic input (Woolley et al., 1997); the locus of both effects is postsynaptic. More recently, it has been shown that the increase in spine density of hippocampal neurons is due to an indirect effect: estrogen regulated reduction of inhibitory inputs (Murphy et al., 1998). In the *X. laevis* larynx, the observation that spontaneous postsynaptic potentials are the same in the sexes argues instead for a presynaptic locus for sexually differentiated function: More transmitter is released from the terminals of female than male laryngeal motor neurons (Tobias et al., 1995). If endogenous estrogen is responsible for sexual differentiation of synaptic strength, it must act either directly on the motor neuron to change the probability of transmitter release or via a retrograde signal from the laryngeal muscle fiber. Laryngeal motor neurons of adult females do not accumulate radioactive estrogen (Morrell et al., 1975), but a number of molecular approaches (polymerase chain reaction as well as Northern and RNase protection assays) suggest that laryngeal muscle does express a classical estrogen receptor (Wu et al., 1997) and thus might provide a retrograde signal to the presynaptic cell.

Estrogen modulation of synaptic efficacy is of considerable clinical interest because estrogen can enhance cognitive function in young women (Sherwin, 1998) and postmenopausal hormone replacement therapy is associated with delayed onset of Alzheimer disease (Kawas et al., 1997; Tang et al., 1996). Further insight into mechanisms for estrogen action will help us to under-

stand the molecular basis for the activational effects of this hormone on neural function.

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