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Ontogenesis of Gonadotropin-Releasing Hormone in the Human Fetal Hypothalamus (40793)

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The human fetal pituitary is capable of producing gonadotropin as early as 9–10 weeks fetal age (1, 2). Although a direct vascular connection between the hypothalamus and the anterior pituitary gland may not become established until as late as 18–20 weeks of fetal age (3, 4), the finding of immunoreactive gonadotropin-releasing hormone (GnRH)-like activity in the hypothalamus as early as 6–8 weeks of fetal age (5, 6) suggests the possibility that pituitary gonadotropin secretion is under hypothalamic regulation from its inception.

There is a clear sex difference in fetal pituitary gonadotropin secretion during the second trimester of pregnancy (1, 2, 7). Circulating levels of both FSH and LH are considerably higher in female than in male fetuses. This sex difference presumably results from secretions by the fetal testis (8); if these act to suppress gonadotropin secretion at the hypothalamic level, this should be reflected by a sex difference in hypothalamic GnRH content.

The presence of immunoreactive GnRH-like material in the fetal hypothalamus has been confirmed recently by a number of workers (5, 6, 9–12). However, interpretation of these data has been difficult because of major differences in the amounts measured among various laboratories. This variability might be explained by differences in the selection of specimens, in their processing, or in the specificity of the assay procedures used. Tissue processing is of particular importance, both because proteolytic degradation of GnRH in tissue is rapid (13) and because GnRH may exist in the hypothalamus in a protein-bound complex (14).

The present study was designed to assess the effects of different storage and extraction procedures on GnRH determinations and to document whether the immunoreactive GnRH activity corresponds to the intact decapeptide hormone. The methods developed were then utilized to determine hypothalamic GnRH content in previable human fetuses during the period of sexual differentiation in order to clarify the relationship of GnRH to pituitary gonadotropin secretion at this time.

Materials and methods. Fifty-eight fetal specimens of ages ranging from 8–17.5 weeks[1] were obtained by hysterotomy performed because of gynecosocial indications on otherwise healthy pregnant women. Sex was verified by karyotype and/or gonadal histology in the smaller fetuses, and by inspection of the external and internal genitalia in those over 5 cm crown–rump length (16).

The brain was exposed and removed from the skull. Hypothalamic specimens were obtained through an incision which started anteriorly just behind the optic chiasm and continued medial to the optic tracts, and then posterior to the mamillary bodies. This area was excised to procure a tissue block about 4 mm in depth. Wet weights varied from 20–125 mg. In 33 cases additional brain specimens, weighing from 70 to 430 mg, were excised from one of the occipital lobes. After being weighed, the tissue blocks were frozen (−20°) either directly or in 2 ml of 0.1 *N* HCl within 20–30 min of fetal delivery.

At the time of assay, the acid–frozen specimens (n = 44) were thawed and homogenized (in acid) on ice in a glass homogenizer. Homogenization of speci-

[1] Fetal age was derived from crown–rump length data according to Patten (15).

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mens fresh–frozen without acid was carried out either in 2 ml 0.1 *N* HCl ($n = 6$) or in 2 ml 0.01 *M* phosphate-buffered saline (PBS), pH 7.4 (the RIA assay buffer) ($n = 8$), as above. Homogenates were neutralized with 0.1 *N* NaOH and centrifuged at 3000 rpm for 20 min at 4°C, after which the supernatants were collected for radioimmunoassay.

Recovery of GnRH was assessed after the addition of either 50 and 200 pg synthetic GnRH (Ayerst Laboratories) or 23,000 cpm ^{125}I-GnRH to 50–75 mg blocks of acid–frozen cerebral cortex, in which the endogenous GnRH concentration averaged 0.17 pg/mg. The GnRH was added to the tissue blocks either prior to freezing in acid and homogenization or to the supernatants following centrifugation.

GnRH was measured by radioimmunoassay using a double antibody system, as described by Nett *et al.* (17), with minor modifications. Purification of the radiolabeled hormone was performed on a Sephadex G-25 column (30 × 0.6 cm) equilibrated with 0.01 *M* acetic acid. The free iodide was eluted with this acid; addition of gelatin to the eluant acid in a final concentration of 0.1% resulted in a more discrete ^{125}I-GnRH peak, the descending limb of which showed the greatest immunoreactivity. ^{125}I-GnRH was aliquoted and stored at −20°; it showed no loss of immunoreactivity for up to 4 months. The GnRH antiserum (R42, kindly provided by Drs. T. Nett and G. Niswender) previously well characterized (10, 17–19), was used at a final dilution of 1:1,000,000: It was preincubated with synthetic GnRH standard (1–30 pg) or brain homogenate for 24 hours at 4°C. After addition of the radiolabeled GnRH (5–10,000 cpm), the reaction was allowed to proceed for a further 2–3 days prior to the addition of the second stage (goat anti-rabbit gamma globulin plus carrier normal rabbit serum). The assay sensitivity was such that 1 pg GnRH per assay tube could be readily detected. One hypothalamic homogenate was used as an interassay "control"; values for this sample ranged from 4.61 to 6.38 ng GnRH over a 3-year period (coefficient of variation ± 11.2%, $n = 13$) without a trend toward diminished values with storage.

Since each of the hypothalamic specimens included a variable amount of extrahypothalamic neural tissue, results were expressed as total hypothalamic content rather than as concentration. Differences between groups were evaluated by the Wilcoxon–Whitney–Mann test (20).

Ion-exchange chromatography was performed on a column (1 × 12 cm) packed with carboxymethylcellulose (CMC) (CM52, Pharmacia) equilibrated in 0.1 *M* ammonium acetate, pH 6.6 (21). A hypothalamic extract (from a male, 13.8 weeks) and synthetic GnRH extracted in the same fashion with 0.1 *N* HCl and then neutralized with 0.1 *N* NaOH were applied to the column in two separate runs and eluted with the same ammonium acetate buffer. One-milliliter fractions were collected and assayed for immunoreactivity.

Isoelectrofocusing was performed on two fetal (one male, 15 weeks; one female, 17 weeks) hypothalamic extracts on preprepared PAGE plates using the LKB wide-range (pH 3.5–9.5) Ampholine buffer system and multiphor apparatus (22). The samples, with synthetic GnRH markers on either side, were applied at the anode and allowed to run for 2 hr at 4°C (power of 4 W). On completion, the gel was cut into approximately 5-mm slices, and the hormone eluted in 1 ml 0.1% gelatin PBS by shaking overnight at 4°C. The eluant of each slice was assayed for immunoreactivity.

Results. Hypothalamic specimens which were fresh–frozen without prior acidification showed considerably lower (or undetectable) amounts of GnRH compared to acid–frozen specimens of comparable ages (Table I). Accordingly, only data from the acid-frozen specimens ($n = 44$) were used.

Although acid homogenization of fresh–frozen specimens seemed superior to buffer homogenization the difference between the two groups (Table I, all ages) did not reach statistical significance ($P = 0.07$).

Recovery of cold or radiolabeled GnRH added to cerebral cortical specimens prior to freezing in acid and homogenization averaged 77.1 ± SD 8.8% ($n = 7$). Recoveries assessed following homogenization and

TABLE I. COMPARISON OF HYPOTHALAMIC GnRH CONTENT ACCORDING TO FETAL AGE AND SEX AND TO METHOD OF EXTRACTION

Extraction method	Hypothalamic GnRH content (ng) mean ± SE (*n*)							
	Acid–frozen acid homogenized (study group)			Fresh–frozen acid homogenized	Fresh–frozen buffer homogenized	Difference *P* value		
Fetal sex	Male	Female	Combined	Combined	Combined	Extraction[a]	Sex[b]	Age
Fetal age (weeks)								
8.0–10.0	0.40 ± 0.17 (4)	0.28 ± 0.12 (5)	0.34 ± 0.09 (9)	< 0.06 (1)	< 0.06 (1)	< 0.05	n.s.*	0.05**
10.1–12.0	0.37 ± 0.07 (3)	0.91 ± 0.20 (10)	0.78 ± 0.17 (13)	0.04 (1)	< 0.06 (1)	< 0.01	n.s.	< 0.001**
12.1–14.0	1.85 ± 0.25 (6)	2.87 ± 0.49 (5)	2.31 ± 0.29 (11)	—	< 0.06 (4)	< 0.001	n.s.	< 0.001**
14.1–17.5	4.70 ± 0.64 (6)	4.70 ± 0.91 (5)	4.70 ± 0.51 (11)	0.44 ± 0.31 (4)	< 0.06, 0.19 (2)	< 0.001	n.s.	
All ages	2.21 ± 0.47 (19)	1.93 ± 0.39 (25)	2.05 ± 0.30 (44)	0.31 ± 0.21 (6)	0.08 ± 0.02 (8)	< 0.001	n.s.	n.s.***

[a] Comparison of fresh–frozen groups (combined) with acid–frozen group.
[b] Comparison of sex difference in acid frozen group.
* n.s. = $P > 0.05$
** Comparison between adjacent age groups (combined) in acid–frozen group.
*** Difference in overall age distribution between sexes in acid–frozen group.

collection of supernatants averaged 101.2 ± SD 10.1% (n = 10). No recovery correction was applied to the hypothalamic data described below.

The immunoreactive material extracted from neural tissue was studied in three different ways to establish identity with synthetic GnRH. First, dose–response slopes for varying volumes of hypothalamic homogenates of sufficient content were parallel to that of the assay standard. Two cortical homogenates of sufficient content also behaved similarly. Second, CMC chromatography of a hypothalamic homogenate gave an identical elution pattern to that of synthetic GnRH when each fraction was assayed for GnRH immunoreactivity (Fig. 1). Third, the isoelectrofocus patterns for two fetal hypothalami were identical to that of the synthetic GnRH markers (Fig. 2).

The content of GnRH in acid–frozen hypothalami prior to 11 weeks fetal age was low (<1 ng) but measurable in all but 1 of 12 specimens (Table I and Fig. 3). GnRH content increased with age ($r = 0.83$, $P < 0.01$ and see Table I) and between 14 and 17.5 weeks values ranged from 2.4 to 6.8 ng. A sex difference was not observed (Table I). A similar trend to increased values with age was also observed in GnRH concentration (data not shown), but, not surprisingly, the data showed considerably more scatter.

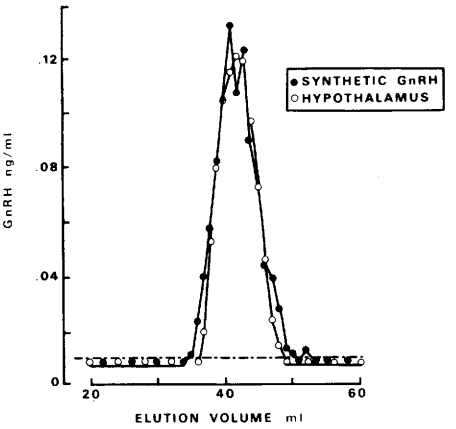

FIG. 2. Isoelectrofocusing patterns of two human fetal hypothalamic extracts and of synthetic GnRH plotted as mass of GnRH (ng) measured/ml eluant against the number of gel slices (see text for details). The dotted line is the limit of assay sensitivity for this experiment (5 pg/ml eluant).

GnRH was low or undetectable, i.e., less than 1 pg/mg wet weight in 31 of 33 cerebral cortical homogenates; the remaining two showed lower concentrations (3.0 and 3.5 pg/mg) than all but two of the hypothalamic specimens.

Discussion. The present report confirms that the human fetal hypothalamus contains immunoreactive GnRH. Although conclusive proof of identity was not obtained, the

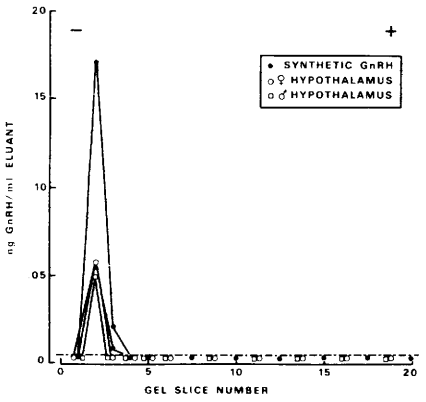

FIG. 1. Elution patterns of immunoreactive GnRH of a human fetal hypothalamic extract and of synthetic GnRH on carboxymethylcellulose chromatography. The dotted line is the limit of assay sensitivity for this experiment.

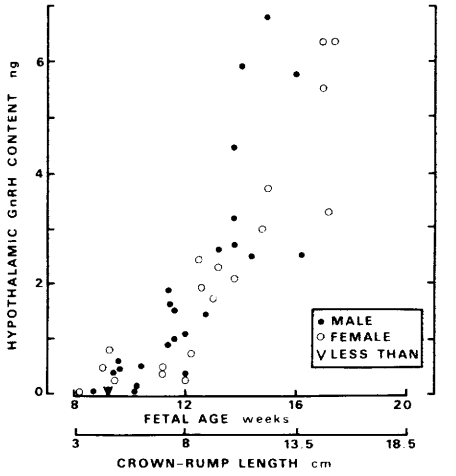

FIG. 3. Fetal human hypothalamic GnRH content (ng) expressed as a function of fetal age (weeks) or crown–rump length (cm) and sex.

immunoreactive material was found to be indistinguishable from the synthetic decapeptide based upon the following observations. First, the hypothalamic extracts showed dose–response slopes parallel to that of synthetic GnRH in the RIA. The RIA employed has been well characterized and appears to be virtually specific for the intact decapeptide, with the exception of a small amount of cross-reactivity for the 2–10 fragment (18, 19), a substance which may be a biological breakdown product of GnRH (21, 23). Second, the behavior of one hypothalamic extract on CMC ion-exchange chromatography, which can separate the intact decapeptide from the nona-, octa-, hepta-, and hexafragments (21), was identical to that of synthetic GnRH (Fig. 1). Finally, the physicochemical identity of the immunoreactive material and of synthetic GnRH was confirmed by isoelectric focusing (Fig. 2). Previous reports have demonstrated that human fetal hypothalamic extracts possess biologic activity by their ability to release gonadotropins both *in vivo* (11) and *in vitro* (24).

It is obvious that differences in the handling of neural tissues can markedly influence the measurement of their GnRH content. Specimens must be frozen immediately in acid in order to maximize recovery of GnRH (Table I). It is not clear at present whether the acid pH serves to inhibit enzymatic degradation of GnRH (13) or to cleave it from a tissue-binding protein (14), thus enhancing recovery. Using these conditions, the endogenous hormone is stable, as is shown by the interassay coefficient of variation of 11.2% over a 3-year period, with no trend toward diminished values with time, despite repeated thawing and refreezing.

There are a number of differences in the present study from others in the literature (Table II) which may serve to explain apparent discrepancies in the results obtained. Some studies have included material from spontaneous or induced abortions (10) or have delayed dissection and freezing of tissues for several hours (12). Although the rate of enzymatic degradation of GnRH in fetal neural tissues *in vivo* and *in vitro* is not known (28), such delays may reduce recovery. Other studies (5, 6) have reported only GnRH concentration data, a parameter which is obviously open to variable error, given the poorly defined anatomical boundaries of the fetal hypothalamus. The low levels of hypothalamic GnRH found in several reports (6, 10, 11) and the failure to detect GnRH in 25% of specimens in one report (10), probably reflect low rates of GnRH recovery, since in these studies the tissue was fresh–frozen without acid. Finally, although some studies (5, 10) have used the same, highly specific, GnRH antibody (R42) as in the present report, others (6, 11, 12) have utilized less specific C-terminal assays which cross-react with several cleavage products of the GnRH decapeptide (27, 29). The present report is the first to present data documenting the physicochemical identity with synthetic GnRH of the assayed material. Thus, a lack of assay specificity may explain the threefold higher GnRH values reported by Siler-Khodr and Khodr (12) at comparable fetal ages.

The principal aim of the current study, following initial validation of the methods used, was to relate the ontogenetic pattern of hypothalamic GnRH with what is known regarding fetal pituitary gonadotropin secretion. Clearly, hypothalamic GnRH is detectable by as early as 8 weeks of fetal age and, therefore, precedes or coincides with the onset of pituitary gonadotropin secretion (1, 2). Furthermore, hypothalamic GnRH content rises between 11 and 17.5 weeks in parallel with fetal pituitary gonadotropin secretion at this time (1, 2, 7). Whether or not there is a later decline in hypothalamic GnRH in parallel with the decrease in gonadotropin secretion after midgestation (1, 2, 7) cannot be determined from the present study; however, such a decline in bioassayable hypothalamic GnRH toward term has been reported (24). Taken together, these observations suggest that the fetal pituitary may be under hypothalamic regulation from its inception. However, the route by which GnRH reaches the pituitary at this time remains undefined, since it has been reported that a definitive portal system connecting the hypothalamus and pituitary may not be-

TABLE II. SUMMARY OF STUDIES OF HYPOTHALAMIC GnRH CONTENT AND CONCENTRATION IN THE HUMAN FETUS

Source of material	Extraction method	Radioimmunoassay method	Fetal age (weeks)	No. of specimens	Hypothalamic GnRH		Reference
					Content (ng)[a]	Concentration (pg/mg)[a]	
Therapeutic abortion (hysterectomy); normal pregnancies	Immediate dissection and freezing; methanol homogenization	Nett–Niswender R42 (17)	8–22	10	—	10.7 (3–66)	Winters *et al.* 1974 (5)
Therapeutic abortion (method?)	Fresh frozen after dissection; lyophilized; acetone, acetic acid, boiled and relyophilized	Nett–Niswender R42 (17)	11–22.5	44	0.65 (n.d.–20)[b]	0.70 (n.d.–13)	Aubert *et al.* 1977 (10)
Therapeutic abortion (hysterectomy)	Fresh–frozen after dissection; methanol homogenization	Dermody #173 (25)	9–20	15	—	1.18 (0.1–29)	Aksel and Tyrey 1977 (6)
Therapeutic abortion (hysterotomy); normal pregnancies	Immediate dissection and freezing; acid homogenization	Jeffcoate (26)	11–19	8	0.17[c]	1.58 (0.6–11.8)	Gilmore *et al.* 1978 (11)
Therapeutic abortion, spontaneous abortion and stillbirth	Dissection within 12 hr; frozen in acetone; bicarbonate buffer homogenization	Siler-Khodr and Khodr (27)	14–38	45	~14.5 (11–24)	~ 50 (15–140)	Siler-Khodr and Khodr 1978 (12)
Therapeutic abortion (hysterotomy); normal pregnancies	Immediate dissection and acid freezing; acid homogenization	Nett–Niswender R42 (17)	8–17.5	44	1.56 (n.d.–6.8)	26.1 (n.d.–75.6)	Present paper

[a] Median (range).
[b] n.d. = nondetectable.
[c] Mean; previously reported by Mortimer *et al.* 1976 (9).

come established until 18–20 weeks of fetal age (3, 4).

The absence of a sex difference in hypothalamic GnRH content is in sharp contrast to the finding of considerably higher circulating levels of FSH and LH in female than in male fetuses at this time (1, 7). If hypothalamic GnRH content reflects GnRH secretion, these observations would point to a sex-specific influence upon gonadotropin secretion at the pituitary level. Thus, it seems likely that modulation of the pituitary response to GnRH by some testicular secretory product (8) occurs during this stage of fetal life. On the other hand, it remains possible that a sex difference in GnRH content does exist in specific loci within the hypothalamus which are responsive to feedback regulation by this putative testicular factor but which escaped detection by measurements of total hypothalamic content.

The finding of low or undetectable levels of GnRH in extracts of cerebral cortex confirms previous observations. The identity of these small amounts of immunoreactive material was not rigorously examined by chromatographic or electrofocusing techniques; although dose–response slopes of some cortical extracts were parallel to that of the assay standard in the current study, this was not the case in another study (6), and, therefore, one cannot exclude the possibility that this material represents some sort of artifact.

Summary. Gonadotropin-releasing hormone (GnRH) was measured by radioimmunoassay in 44 human fetal hypothalamic and 33 cerebrocortical specimens of 8–17.5 weeks fetal age. These specimens, obtained within 30 min of hysterotomy, were frozen in 0.1 *N* HCl and subsequently homogenized and extracted in acid. Attempts to extract GnRH in additional hypothalamic specimens under either neutral ($n = 6$) or acid ($n = 8$) conditions from tissue frozen without acid resulted in considerably lower values; accordingly, these data were rejected. All hypothalamic and cerebral specimens in which sufficient GnRH was detected exhibited dose–response slopes parallel to that of synthetic GnRH. Furthermore, the immunoreactive material from hypothalamic extracts was indistinguishable from synthetic GnRH in its elution pattern on carboxymethylcellulose ion-exchange chromatography and in its mobility during isoelectric focusing. Prior to 11 weeks of fetal age, the hypothalamic content of GnRH was low (<1 ng) but increased progressively to values ranging from 2.4 to 6.8 ng between 14 and 17.5 weeks. No sex difference was observed. The parallelism between this rise in hypothalamic GnRH content and previously reported changes in pituitary and serum gonadotropin concentrations in the human fetus suggests that the anterior pituitary may be under hypothalamic regulation from its inception. The absence of a sex difference in hypothalamic GnRH content despite clearly higher circulating FSH and LH levels in females vs males at comparable ages suggests that the putative fetal testicular gonadotropin feedback inhibitor is operative at the pituitary rather than at the hypothalamic level.

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1. Clements, J. A., Reyes, F. I., Winter, J. S. D., and Faiman, C., J. Clin. Endocrinol. Metab. 42, 9 (1976).
2. Kaplan, S. L., and Grumbach, M. M., Acta Endocrinol. (Copenhagen) 81, 808 (1976).
3. D'Espinasse, P. G., J. Anat. 68, 11 (1933).
4. Niminieva, K., Acta Paediatr. Scand. 39, 366 (1949).
5. Winters, A. J., Eskay, R. L., and Porter, J. C., J. Clin. Endocrinol. Metab. 39, 960 (1974).
6. Aksel, S., and Tyrey, L., Fertil. Steril. 28, 1067 (1977).
7. Takagi, S., Yoshida, T., Tsubata, K., Ozaki, H., Fujii, T. K., Nomura, Y., and Sawada, M., J. Steroid. Biochem. 8, 609 (1977).
8. Reyes, F. I., Boroditsky, R. S., Winter, J. S. D., and Faiman, C., J. Clin. Endocrinol. Metab. 38, 612 (1974).
9. Mortimer, C. H., McNeilly, A. S., Rees, L. H., Lowry, P. J., Gilmore, D., and Dobbie, H. G., J. Clin. Endocrinol. Metab. 43, 882 (1976).

10. Aubert, M. L., Grumbach, M. M., and Kaplan, S. L., J. Clin. Endocrinol. Metab. **44**, 1130 (1977).
11. Gilmore, D. P., Dobbie, H. G., McNeilly, A. S., and Mortimer, C. H., J. Reprod. Fert. **52**, 355 (1978).
12. Siler-Khodr, T. M., and Khodr, G. S., Amer. J. Obstet. Gynecol. **130**, 795 (1978).
13. Koch, Y., Baram, T., Chobsieng, P., and Fridkin, M., Biochem. Biophys. Res. Commun. **61**, 95 (1974).
14. Shin, S. H., and Howitt, C., Neuroendocrinology **24**, 14 (1977).
15. Patten, B. M., "Human Embryology," 3rd. ed., p. 142. McGraw-Hill, New York (1968).
16. Jirasek, J. E., "Development of the Genital System and Male Pseudohermaphroditism," p. 10, The Johns Hopkins Press, Baltimore and London (1971).
17. Nett, T. M., Akbar, A. M., Niswender, G. D., Hedlund, M. T., and White, W. F., J. Clin. Endocrinol. Metab. **36**, 880 (1973).
18. Jonas, H. A., Burger, H. G., Cumming, I. A., Findlay, J. K., and deKretser, D. M., Endocrinology **96**, 384 (1975).
19. Dahlén, H. G., Voigt, K. H., and Schneider, H. P. G., Horm. Metab. Res. **8**, 61 (1976).
20. Snedecor, G. W., and Cochran, W. G., "Statistical Methods," 6th ed., p. 130. Iowa State Univ. Press, Ames (1967).
21. Jeffcoate, S. L., and Holland, D. T., Acta Endocrinol. (Copenhagen) **78**, 232 (1975).
22. Karisson, C., "Multiphor-Analytical Gel Electrofocusing in Polyacrylamide," (L.K.B. Technical Bulletin No. 2117). Applications Laboratory L.K.B., Stockholm (1973).
23. Redding, T. W., Kastin, A. J., Gonzalez-Barcena, D., Coy, D. H., Coy, E. J., Schalch, D. S., and Schally, A. V., J. Clin. Endocrinol. Metab. **37**, 626 (1973).
24. Levina, S. E., *in* "Hormones in Development" (M. Hamburgh, and E. J. W. Barrington, eds.), pp. 547–552. Appleton-Century-Crofts, New York (1971).
25. Dermody, W. C., Becvar, E. A., Windsor, B. L., Wong, A. L., Vaitkus, J. W., Caple, J. E., and Sakowski, R., *in* "Hypothalamic Hormones" (E.S.E. Hafez and J.R. Reel, eds.), pp. 71–99, Ann Arbor Science Publishers, Ann Arbor (1975).
26. Jeffcoate, S. L., Fraser, H. M., Gunn, A., and Holland, D. T., J. Endocrinol. **57**, 189 (1973).
27. Siler-Khodr, T. M., and Khodr, G. S., Amer. J. Obstet. Gynecol. **130**, 216 (1978).
28. Okon, E., and Koch, Y., Nature (London) **263**, 345 (1976).
29. Pique, L., Cesselin, F., Strauch, G., Valcke, J. C., and Bricaire, H., Immunochemistry **15**, 55 (1978).

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