0013-7227/89/1253-1650$02.00/0
Endocrinology

Vol. 125, No. 3
Printed in U.S.A.

Essential Fatty Acid Deficiency Delays the Onset of Puberty in the Female Rat*

SHERYL S. SMITH, MARTHA NEURINGER, AND SERGIO R. OJEDA

Department of Anatomy, Hahnemann University (S.S.S.), Philadelphia, Pennsylvania 19102-1192; and the Division of Neuroscience, Oregon Regional Primate Research Center (M.N., S.R.O.), Beaverton, Oregon 97006

ABSTRACT. This study assessed the effect of a dietary deficiency in the essential fatty acids (EFA) linoleic and linolenic acids on the onset of female puberty. EFA deficiency was produced in female rats by means of a semipurified diet and was biochemically documented by analyzing serum and erythrocyte fatty acid levels of more than 30 fatty acids, including all members of the n-6 and n-3 series. Levels of linoleic acid (18:2 n-6)[1] and all n-6 derivatives, particularly arachidonic acid, were strikingly reduced. A less pronounced but clear-cut decrease in n-3 fatty acids, including docosahexaenoic acid (22:6 n-3) was also found. The times of puberty and first ovulation, as assessed by the ages at vaginal opening and first diestrus, were significantly delayed in EFA-deficient rats. The mechanisms underlying this delay appear to reside at both hypothalamic and ovarian sites. Simulation of preovulatory plasma estradiol (E_2) levels via implantation of E_2-containing Silastic capsules evoked a LH surge 30 h later in control juvenile rats, but not in EFA-deficient animals, indicating a delay in the development of the hypothalamic component of E_2-positive feedback in the latter group. This delay appears to be due at least in part to reduced prostaglandin E_2 (PGE_2) synthesis, as the ability of the neurotransmitter norepinephrine to induce PGE_2 release from median eminence nerve terminals was markedly reduced in EFA-deficient rats compared with that in controls. The decrease in hypothalamic PGE_2 release was related to the EFA deficiency and not to reduced PG synthase activity, as determined by HPLC analysis of PG synthase products derived from exogenous [^{14}C]arachidonic acid. Basal and hCG-stimulated PGE_2 synthesis was also compromised in ovaries from EFA-deficient rats. Depressed gonadal function resulting from the EFA deficiency was further evidenced by a reduced gonadotropin receptor content, a blunted E_2 response to hCG *in vitro*, and an increase in mean serum FSH levels. These results suggest that the delay in puberty resulting from EFA deficiency is due to a reduced availability of arachidonic acid for synthesis of bioactive metabolites. This results in delayed development of both the hypothalamic and ovarian components of the reproductive axis. (*Endocrinology* **125:** 1650–1659, 1989)

FULL maturity of the female reproductive system requires (1) that the hypothalamic-pituitary unit develop the capacity to respond to proestrous levels of estrogen with a LH surge, an ability acquired in the early juvenile period (1, 2); and (2) that production of estradiol (E_2) by the ovary become sufficiently elevated to stimulate gonadotropin release, a condition attained during peripubertal days (3).

A specific group of dietary components known to be required for normal reproductive function are the n-6 essential fatty acids (EFA) linoleic acid and arachidonic acid (4–6). These polyunsaturated fatty acids function as membrane constituents (5–8) as well as precursors for substances such as prostaglandins (PGs), leukotrienes, and epoxyacids (9), which have been implicated in signal transduction mechanisms that operate within the hypothalamus-pituitary-ovarian axis (for reviews, see Refs. 10 and 11). Among these arachidonic acid metabolites, PGE_2 has been shown to be a physiological component in both the processes of LHRH release from the hypothalamus and ovulation (for reviews, see Refs. 12 and 13).

Prolonged dietary deficiency of EFA (both n-6 and n-3 fatty acids) results in irregular estrous cycles, ovulatory failure, sterility, prolonged gestation, and/or impairment of the birth mechanism (4–6). The deficient diet reduces tissue levels of n-6 fatty acids to a much greater extent that those of n-3 fatty acids (14, 15). In turn, all overt signs of EFA deficiency, including reproductive alterations, are prevented by dietary n-6 fatty acids, but not by n-3 fatty acids (6, 8). Thus, the dietary availability of linoleic and arachidonic acids may contribute to the

Received February 6, 1989.
Address all correspondence and requests for reprints to: Dr. Sergio R. Ojeda, Division of Neuroscience, Oregon Regional Primate Research Center, 505 NW 185th Avenue, Beaverton, Oregon 97006.
* Part of this work was performed while two of the authors (S.S.S. and S.R.O) were at the Department of Physiology, University of Texas Health Science Center at Dallas. During this time, S.S.S. was a predoctoral research fellow supported by NIH Training Grant 5T32-HD-07062. This work was supported by NIH Grants HD-09988-Project IV, RR-00163, DK-29930, and NS-25809. This is publication 1666 of the Oregon Regional Primate Research Center.
[1] Fatty acid nomenclature: the first number indicates the length of the carbon chain; the second number (after the colon) specifies the number of double bonds; the third number (after n-) provides the position of the first double bond from the methyl end of the molecule.

timely development of the hypothalamus-pituitary-ovarian system which culminates with the first ovulation.

In an effort to more clearly delineate such a role, we have examined in the present study the effect of EFA deficiency on the development of the reproductive axis and the timing of puberty in the female rat.

Materials and Methods

Animals

Immature female Sprague-Dawley rats, born to rats mated in the lab, were used in all experiments. Their mothers were purchased from either the Holtzman Co. (Madison, WI) or Bantin and Kingman (Freemont, CA). The animals had free access to food and tap water in rooms with a controlled light-dark cycle (14 h on, 10 h off) and constant temperature (23–25 C). Adult female rats (240–300 g), housed individually, were placed on one of three isocaloric diets (see below) 2 weeks before mating; these diets were continued throughout pregnancy and lactation until the day of weaning of the pups (day 21). All litters were adjusted to seven or eight pups/mother on the day of birth. Postweaning pups were then continued on the appropriate diet until puberty (first diestrus), when they were killed. Other groups of rats were killed during development, at 5-day intervals.

EFA-deficient diet

Rats were fed either a regular laboratory stock diet (Ralston-Purina Co., St. Louis, MO) or 1 of 2 semipurified test diets (Teklad, Madison, WI). The stock diet (henceforth referred to as regular diet) contained 4.5% fat derived from animal fat. The diet deficient in EFA (Teklad TD 77052) was identical to the semipurified control diet (Teklad TD 77053), henceforth referred to as control diet) except that it contained medium-chain triglycerides (8–14 carbon fatty acids) instead of corn oil as the source of fat. The deficient diet, therefore, provided no polyunsaturated fatty acids, whereas the control diet contained ample linoleic acid (58% of total fatty acids or 2.9% by weight of the diet). In both cases, fat represented 5% of the diet by weight. Both semipurified diets contained casein (20% by weight), sucrose (68.5%), nonnutritive fiber (20%), and all known essential vitamins (Teklad vitamin mix, 1%) and minerals (William-Briggs, 3.5%).

Determination of serum and erythrocyte fatty acid composition

As biochemical evidence of EFA deficiency, fatty acid composition was determined at 32–34 days of age in serum from two control and two EFA-deficient litters and in erythrocytes from one litter in each group. Lipids were extracted from serum by the procedure of Bligh and Dyer (16) and from red blood cells by the method of Rose and Oklander (17). Total fatty acids were saponified in alcoholic KOH for 1 h, extracted with hexane, and methylated with 14% boron trifluoride-methanol by the method of Morrison and Smith (18). Fatty acid methyl esters were analyzed by gas-liquid chromatography (Perkin-Elmer Sigma 3B, Norwalk, CT) with SP-2330 on a 30-meter glass capillary column (Supelco, Bellefonte, PA). Column conditions were: carrier gas He, 60 cc/min; column temperature, 195 C; flame ionization detector temperature, 250 C; injection port temperature, 250 C. Pure fatty acid standard mixtures were run daily, and each fatty acid was identified by comparing its retention time with that of the known fatty acid in the standard mixture. Fatty acids were quantified by peak area integration with a Hewlett-Packard 3390A integrator (Palo Alto, CA). Thirty-six different fatty acids were reliably resolved by this method, including all members of the n-6 and n-3 series.

Birth statistics

The effects of EFA deficiency on various birth parameters were assessed. These included quantification of pups born, male/female ratio, mortality rate, and body weight of both the pups and mothers. In addition, the deviation from expected date of birth was noted for pups born to normal *vs.* EFA-deficient mothers.

Development and timing of puberty

In one experiment the effects of EFA deficiency on various developmental parameters (body, ovarian, and uterine weights) were monitored at 5-day intervals from days 5–35. Then, to determine the effect of EFA deficiency on the timing of puberty, the animals were inspected daily for vaginal opening (VO) starting on day 29. Body weight was recorded at VO, and thereafter, estrous cyclicity was monitored by daily examination of vaginal cytology. On the day of first diestrus (D_1) after the first estrus, rats were weighed and decapitated, and the ovaries and uterus were dissected out and weighed to the nearest milligram. The presence of corpora lutea was also noted to confirm that ovulation had occurred. Two experiments were performed. In one of them EFA-deficient rats were compared only to animals fed the control diet. In the second experiment a group of rats fed the regular diet was also included.

Pituitary hormones

Serum levels of FSH and PRL were determined at 5-day intervals during development from days 10–35; in addition, serum levels of LH, FSH, PRL, GH, and TSH were determined on D_1. To further characterize alterations in pituitary function, secretory output of FSH, GH, and PRL was assessed in 33-day-old prepubertal rats fed either the control or EFA-deficient diet. Toward this end, sequential blood samples were obtained at hourly intervals over an 8-h period (1000–1800 h) from conscious, freely moving rats implanted with indwelling jugular cannulae; the secretory rates of these hormones were determined by polar planimetry.

In vitro incubation of ovaries

The effect of EFA deficiency on the steroidal responsiveness of the ovary to hCG was also determined. Ovarian halves from 30- to 33-day-old rats were incubated with or without hCG (0.1 IU/ml; APL, Ayerst Laboratories, Rouses Point, NY) at 37 C in a shaking water bath for 3 h under an atmosphere of 95% O_2 and 5% CO_2, as described previously (3, 19). The incubation

medium used was Krebs-Ringer bicarbonate buffer, pH 7.4, containing 1mg/ml glucose and 0.01% BSA. At the end of the incubation, the medium was centrifuged at low speed, transferred to test tubes, and stored at −20 C until assayed for E_2, progesterone (P), and PGE_2.

Incubation of median eminence (ME) fragments

ME fragments were removed from freshly dissected brains using a stereomicroscope, as previously described (20). ME fragments were then incubated (two per flask) at 37 C in 0.5 ml incubation medium. The conditions of the incubation were similar to those previously described (21). The incubation medium contained 10 μg/ml ascorbic acid to reduce oxidation of norepinephrine (NE; see below). Basal release of PGE_2 was measured after an initial 15-min and a subsequent 30-min incubation period. At the end of the latter interval, the medium was replaced, and 60 μM NE was added to all flasks; PGE_2 levels were measured 30 min later by RIA after centrifugation of the spent medium. Two experiments were performed. In the first one, only basal PGE_2 release was measured, comparing EFA-deficient rats with animals fed the control diet. In the second experiment both basal and NE-induced PGE_2 release were measured, comparing EFA-deficient rats with rats fed the regular diet. Since basal PGE_2 release from the ME of EFA-deficient rats was similar in both experiments, the results were combined for purposes of presentation.

E_2-induced LH surge

This study was undertaken to determine whether EFA deficiency altered the development of the estrogen-positive feedback system on LH release, which normally becomes operative in early juvenile rats (2). Thirty-one-day-old late juvenile female rats (fed the control or EFA-deficient diet) were implanted sc with Silastic capsules (Dow-Corning, Midland, MI: id, 0.040 in.; od, 0.085 in.) cut to a length of 20 mm/100 g BW and filled with a solution of E_2 in corn oil (400 μg/ml). The capsules were allowed to incubate overnight at 37 C in 0.1% gelatin-normal saline to stabilize release of the steroid, as previously described (2, 22). Implantation of this type of capsule has been shown to produce circulating E_2 levels of proestrous magnitude and to induce a LH surge within 30 h in late juvenile rats (2). Less mature rats do not respond to this E_2 treatment until 56 h postimplantation. Thirty hours after capsule implantation, the unanesthetized animals were bled at hourly intervals from 1000–1800 h through jugular cannulae implanted 24 h previously (23), and plasma LH levels were determined by RIA.

RIAs

Serum FSH, PRL, GH, and TSH were assessed using NIDDK kits, with minor modifications, and are expressed in terms of the corresponding RP-1 standard provided with the kits. Serum LH was measured by the method of Niswender *et al.* (24), using the RP-1 rat pituitary LH reference preparation, and is expressed in terms of the NIH LH S1 standard. Quantification of E_2 and P released to incubation medium was carried out as previously described (19), using specific antisera obtained from Dr. G. D. Niswender (Colorado State University).

Quantification of PGE_2 released from ME and ovarian fragments was carried out as previously described (21). The specificity of the antiserum used has been reported (25). The sensitivity of the assay was 3.7–7.5 pg/tube.

HPLC separation of arachidonic acid products

To evaluate possible alterations in PG synthase activity in EFA-deficient animals, HPLC profiles of PG produced from [^{14}C]arachidonic acid by the hypothalamus of control and deficient rats were compared, as previously described (26). The hypothalami (two per tube) were homogenized in 500 μl ice-cold 0.1 M phosphate buffer, pH 8.0, containing 0.02 M EDTA. Thereafter, they were incubated in a 1-ml volume with 5.0 μCi [^{14}C]arachidonic acid (3.1 nmol) for 15 min at 37 C. The PG produced were purified in octadecyl silane-bonded silica gel columns before HPLC analysis as previously reported (26).

Measurement of specific hCG- and FSH-binding sites

The effects of EFA deficiency on ovarian LH and FSH receptor content were assessed on days 30 and 35 (hCG/LH receptors) or days 10 and 20 (FSH receptors) in normal and EFA-deficient rats. These ages were chosen because during normal development the most rapid increase in hCG/LH receptors occurs after day 20, whereas FSH receptors increase more rapidly before day 20 (27). The RRA employed has been described in detail previously (27) and is similar to that reported by Richards *et al.* (28). Iodinated hCG and hFSH, with specific activities of 30 and 15 μCi/μg, respectively, were used at almost saturating concentrations for the amount of tissue used. Ovaries were first homogenized in 0.14 M NaCl-0.01 M phosphate buffer, pH 7.4, and crude membrane fractions were prepared by centrifugation at 30,000 × *g* for 15 min. Rehomogenized membrane fractions, prepared from 1–2 mg wet wt tissue, were incubated overnight with 80,000–100,000 cpm labeled ligand in a total volume of 120 μl at 24 C in a shaking water bath. The binding reaction was terminated with the addition of cold buffer and centrifugation of the membrane-bound ligand. Specific binding was calculated by subtracting nonspecific binding (obtained in the presence of an excess of unlabeled hormone) from binding in the absence of unlabeled hormone. Results are expressed as counts per min bound/μg DNA. The DNA content of the pellet was assessed using the method of Burton (29).

Statistical methods

Differences between two diet groups were analyzed using Student's *t* test. When more than two diet groups were compared, the differences were analyzed with a one-way analysis of variance and Dunnett's test for comparing a control mean to each other group mean. In addition, the Mann-Whitney U nonparametric procedure was used to determine statistical differences in hCG-stimulated E_2 release from ovaries of control *vs.* EFA-deficient groups *in vitro*. This test was implemented due to the heterogeneity of variance (Bartlett's test) exhibited by data from control ovaries. Analysis of hormone secretory episodes was carried out using Student *t* test.

Results

Fatty acid composition

The presence of a biochemical EFA deficiency was documented by the fatty acid analysis of serum and erythrocytes (Table 1) in late juvenile 32- to 34-day-old rats fed a control or EFA-deficient diet. Levels of linoleic acid (18:2 n-6) and all of its n-6 derivatives were greatly reduced in the EFA-deficient group. Most notably, arachidonic acid (20:4 n-6) was reduced by 90% in serum and by 50% in erythrocytes. Levels of eicosatrienoic acid (20:3 n-9), a specific marker of EFA deficiency, were increased 38 times above control values in serum and over 150 times in erythrocytes. This abnormal fatty acid partially replaces arachidonic acid in EFA deficiency. The ratio of eicosatrienoic to arachidonic acid has frequently been used as an index of EFA deficiency (6), with values above 0.1–0.4 considered indicative of a deficiency state. This ratio was 0.018 and 0.003 in control serum and erythrocytes, respectively, whereas in the EFA-deficient group it rose to 7.45 in serum and 1.2 in erythrocytes (Table 1).

Developmental parameters

Body weights of pups born to EFA-deficient mothers were significantly lower ($P < 0.02$) than those of pups born to mothers fed either the control or the regular diet (Table 2). Although mortality rate and the male/female ratio appeared to be increased in pups born to EFA-deficient mothers, these differences were not statistically significant.

Body weights of EFA-deficient pups were also significantly lower ($P < 0.05$) than those of pups fed either the control or the regular diet throughout development (days 5–35), as were uterine (days 10–35) and ovarian weight (days 10–35; Fig. 1). These differences were significant at each age, with the exception that ovarian weight was not significantly different between the groups on day 25. Maximal differences in uterine weight ($P < 0.01$) were noted during the late prepubertal period (days 30–35), a time during which there was a marked increase in the uterine weights of rats fed the control and regular diets.

Time of puberty

The time of puberty was significantly delayed ($P < 0.005$) in EFA-deficient rats, as assessed by either age at the time of VO or the first D_1 in two separate experiments (Table 3). Surprisingly, rats fed the control diet exhibited VO and D_1 vaginal cytology at a later age (by 4 days; $P < 0.001$) than rats fed the regular diet. Although EFA-deficient rats were older than the two control groups at both VO and D_1, their body weight was not significantly different from that of controls on either of these 2 days, reflecting the reduced rate of body weight gain observed during postnatal development in EFA-deficient animals (Fig. 1). In all cases, rats killed on D_1 had ovulated, as indicated by the presence of corpora lutea in the ovarian tissue. At this time weights of the ovaries and uteri from deficient rats were significantly reduced ($P < 0.05$ and $P < 0.01$, respectively) compared with those in the group fed the control diet. However, a significant difference in these parameters was only seen in the second experiment.

TABLE 1. Serum and erythrocyte fatty acids (percentage of total fatty acids)

Fatty acid	Serum		Erythrocytes	
	Control (n = 11)	EFA-deficient (n = 11)	Control (n = 6)	EFA-deficient (n = 6)
18:2 n-6 (linoleic)	18.3 ± 0.4	1.4 ± 0.1[a]	7.9 ± 0.2	1.0 ± 0.1[a]
20:4 n-6 (arachidonic)	23.8 ± 1.0	2.3 ± 0.2[a]	25.4 ± 1.4	12.8 ± 0.6[a]
Total n-6	46.8 ± 1.1	5.5 ± 0.5[a]	40.8 ± 1.6	17.4 ± 0.5[a]
22:6 n-3 (docosahexaenoic)	0.7 ± 0.1	0.1 ± 0.1[a]	1.5 ± 0.2	2.3 ± 0.4[b]
Total n-3	1.2 ± 0.2	0.6 ± 0.4[c]	2.1 ± 0.2	3.2 ± 0.5[b]
Total EFA (n-6 + n-3)	48.0 ± 1.0	6.2 ± 0.5[a]	42.9 ± 1.6	20.6 ± 0.8[a]
18:1 n-9 (oleic)	19.3 ± 0.8	38.6 ± 0.9[a]	13.4 ± 0.4	22.8 ± 0.6[a]
20:3 n-9 (eicosatrienoic)	0.4 ± 0.1	15.2 ± 1.5[a]	0.1 ± 0.04	15.4 ± 0.9[a]
20:3 n-9/20:4 n-6	0.018 ± 0.003	7.45 ± 1.23[a]	0.003 ± 0.002	1.20 ± 0.03[a]

Values are the mean ± SEM.
[a] $P < 0.0005$ *vs.* control diet.
[b] $P < 0.025$ *vs.* control diet.
[c] $P < 0.005$ *vs.* control diet.

TABLE 2. Effect of a maternal EFA deficiency on some vital parameters of the pups at birth

Groups	Deviation from expected date of birth (days)	No. of pups/litter	Mortality rate (no. of dead/ total)	No. of males/ females	BW of pups (g)[a]	BW of dams (g)
Control diet (3 litters)	0.67 ± 0.33	12.7 ± 1.2	0.33 ± 0.33	1.02 ± 0.27	6.22 ± 0.11	320.7 ± 9.7
EFA-deficient (10 litters)	0.90 ± 0.02	10.5 ± 0.86	0.60 ± 0.27	1.76 ± 0.34	5.30 ± 0.16[b]	343.1 ± 11

Values are the mean ± SEM. An EFA-deficient diet was provided to the dams 1 week before fertilization. The date of fertilization was determined by the appearance of sperm in the vaginal lavage after exposure to male rats.

[a] These values represent the mean of the litter means.

[b] $P < 0.02$ *vs.* control diet.

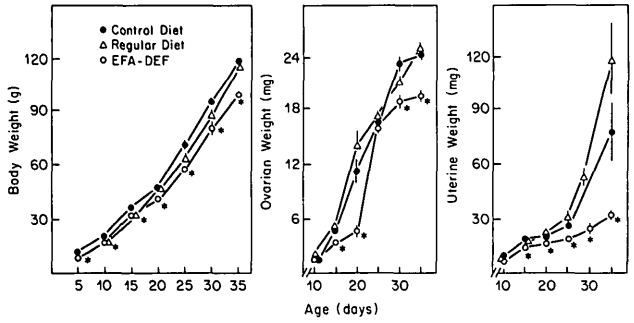

FIG. 1. Growth parameters assessed during postnatal development of female rats fed a semipurified control diet (Teklad TD 77053), a regular diet (Purina cat chow), or a diet deficient in EFA (Teklad TD 77052). For this and the following figures, *vertical lines* represent the SEM. Each *point* represents the mean of 10–15 animals. EFA-DEF, EFA-deficient diet. *, $P < 0.05$ *vs.* control and regular diets.

Development of E_2 positive feedback

It has been previously shown that simulation of first proestrous levels of E_2 induces a LH surge within 30 h in late juvenile rats, but fails to do so in animals younger than 28 days of age, which respond to the E_2 challenge only after 56 h(2). In the present study 31-day-old EFA-deficient rats failed to show LH surge after 30 h of exposure to E_2 (Fig. 2). In marked contrast, in four of five age-matched rats fed the control diet, serum LH levels rose from negligible levels to 33 ng/ml after a similar exposure to E_2.

Pituitary function

When measured on D_1 there were no significant differences in the plasma levels of LH, FSH, PRL, or GH among EFA-deficient, control, and regular diet groups (not shown). Plasma TSH levels, on the other hand, were lower ($P < 0.05$) in animals fed a regular diet (154 ± 13 ng/ml) than in rats fed either a control (273 ± 26 ng/ml) or an EFA-deficient diet (246 ± 14 ng/ml). When examined during selected days of development, serum FSH levels were generally higher and PRL levels lower in EFA-deficient rats compared with those in either control group (Fig. 3). Examination of the secretory output of FSH, GH, and PRL over an 8-h period on day 33 revealed that the secretory rate of FSH, but not that of PRL or GH, was significantly higher ($P < 0.05$) in EFA-deficient rats than in animals fed the control diet (Table 4). Although the secretory rate of PRL tended to be lower in EFA-deficient than in rats fed the control diet the differences did not reach statistical significance because of the large variability observed in the control group.

TABLE 3. Effect of EFA deficiency on the time of puberty in female rats

Groups	No. of litters	No. of rats	Age at VO (days)	BW at VO (g)	Age at D_1 (days)	BW at D_1 (g)	Ovarian wt (mg)	Uterine wt (mg)	CL-positive (no./total)
Exp 1									
Control diet	3	13	37.8 ± 0.5	126.1 ± 2.9	39.3 ± 0.5	135.4 ± 2.0	32.7 ± 1.5	107.7 ± 4.0	13/13
EFA-deficient	10	36	41.3 ± 0.7[a]	127.2 ± 2.3	42.2 ± 0.6[c]	132.7 ± 2.5	32.5 ± 0.8	95.3 ± 2.9	36/36
Exp 2									
Control diet	2	10	38.3 ± 1.2	128.4 ± 5.2	40.4 ± 1.0	132.0 ± 5.4	43.6 ± 2.8	121.8 ± 6.8	10/10
Regular diet	3	15	34.1 ± 0.4[b]	132.1 ± 4.6	35.9 ± 0.4[b]	138.1 ± 4.3	41.1 ± 3.5	133.4 ± 3.1	15/15
EFA-deficient	3	11	45.9 ± 0.8[a]	126.5 ± 3.2	47.2 ± 0.8[a]	129.7 ± 3.1	36.1 ± 1.3[c]	95.7 ± 2.9[d]	11/11

Values are the mean ± SEM. EFA deficiency was initiated 1 week before the day of fertilization and continued throughout pregnancy and postnatal development of the pups.

[a] $P < 0.005$ *vs.* control diet.

[b] $P < 0.001$ *vs.* control diet.

[c] $P < 0.05$ *vs.* control diet.

[d] $P < 0.01$ *vs.* control diet.

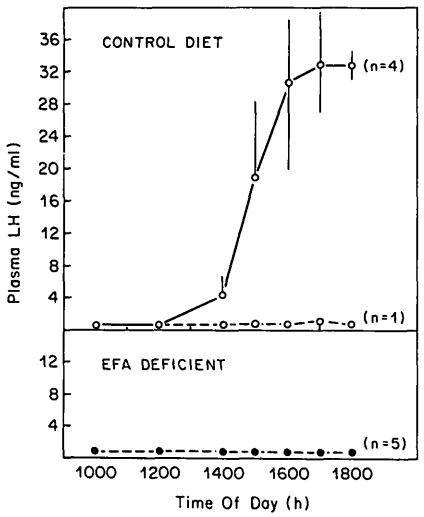

FIG. 2. Differential ability of E_2 to induce a LH surge in prepubertal (31-day-old) female rats fed a control (*upper panel*) *vs.* an EFA-deficient (*lower panel*) diet. Animals were implanted the previous day (−30 h) with Silastic capsules (20 mm/100 g BW) filled with a solution of E_2 in corn oil (400 μg/ml). Of five control rats, one animal failed to respond to the E_2 treatment with a LH surge.

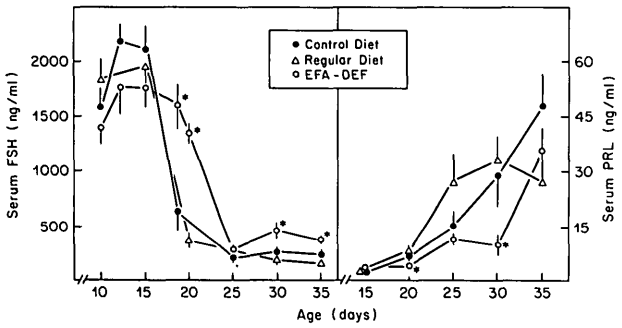

FIG. 3. Developmental changes in serum FSH and PRL levels in female rats fed a control, regular, or EFA-deficient (EFA-DEF) diet. Each *point* represents the mean of 8–10 animals. *, $P < 0.05$ *vs.* control and regular diets.

TABLE 4. Effect of EFA deficiency on secretory output of anterior pituitary hormones

Groups	No. of rats	Secretory rate (ng/ml·h)[a]		
		FSH	PRL	GH
Control diet	4	120.0 ± 10.5	9.2 ± 4.1	25.0 ± 4.6
EFA-deficient diet	5	158.7 ± 12.1[b]	3.7 ± 0.8	22.7 ± 3.7

[a] Blood samples were obtained every hour for 8 h. Hormone levels were measured by RIA, values were plotted, and the areas under the curve were measured with a polar planimeter. Values given are the mean ± SEM.

[b] $P < 0.05$ *vs.* control diet.

PGE_2 release

Release of PGE_2 from ovarian and ME fragments was assessed to determine whether formation of this arachidonic acid metabolite was altered in EFA-deficient rats. As shown in Fig. 4 (*right panel*), incubation with hCG at

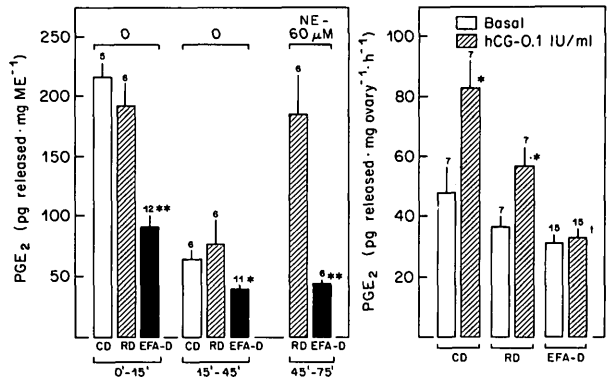

FIG. 4. PGE_2 released *in vitro* from ME nerve terminals (*left panel*) or ovaries (*right panel*) from 30- to 33-day-old rats fed a control (CD), regular (RD), or EFA-deficient (EFA-D) diet in response to NE (ME) or hCG (ovaries). Within each incubation group: *left panel*, *, $P < 0.05$ *vs.* RD or CD; **, $P < 0.002$ *vs.* RD or CD; *right panel*, *, $P < 0.05$ *vs.* basal release; †, $P < 0.005$ *vs.* RD or CD (hCG-stimulated).

a dose of 0.1 IU/ml failed to stimulate PGE_2 release from ovaries of EFA-deficient animals, whereas ovaries from both the control and regular diet groups responded to the gonadotropin with a significant ($P < 0.05$) increase in PGE_2 release.

In addition, both basal and NE-stimulated release of PGE_2 from ME fragments of EFA-deficient animals were markedly lower than control or regular diet group values (Fig. 4, *left panel*). The greatest difference was observed during the first 15-min incubation period and after exposure to NE, times at which PGE_2 levels in the deficient rats were 50–75% lower than control or regular diet values ($P < 0.002$). In contrast to MEs from deficient animals, MEs from the group fed the regular diet more than doubled PGE_2 release in the presence of NE.

Hypothalamic synthesis of PGs

To assess the activity of PG synthase in the hypothalamus of EFA-deficient and control rats fed the control diet, the formation of PGs from [^{14}C]arachidonic acid in hypothalamic homogenates was examined by HPLC analysis of the products. As depicted in Fig. 5, EFA deficiency altered neither the spectrum nor the relative amounts of PGs formed from exogenous arachidonate. Although PGD_2 formation appeared to be slightly increased in EFA-deficient rats, the radioactivity associated with the PGD_2 peak was very similar in both groups (5884 and 5623 cpm in control and EFA-deficient groups, respectively).

Ovarian gonadotropin receptors

FSH receptor levels measured in ovaries from EFA-deficient rats were markedly lower than control or regular diet values ($P < 0.001$) when assessed on day 20 (Fig. 6, *left panel*). However, on day 10 when normal values

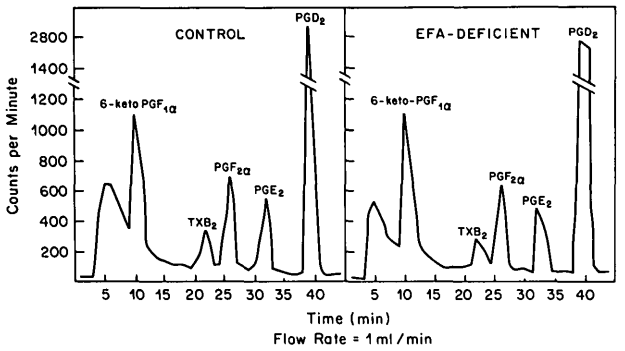

FIG. 5. HPLC elution profiles of PG and thromboxane-B_2 (TXB_2) produced from [^{14}C]arachidonic acid by hypothalamic homogenates of prepubertal female rats fed a control (*left panel*) or an EFA-deficient (*right panel*) diet.

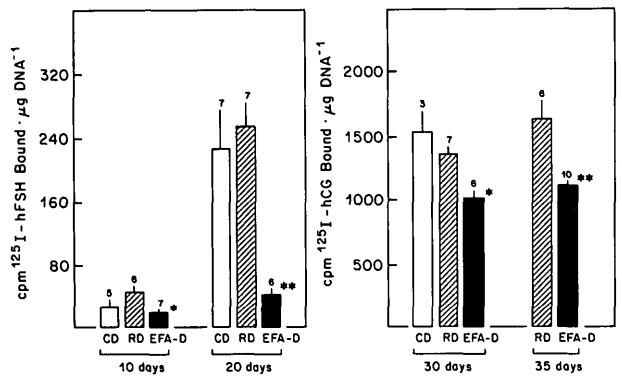

FIG. 6. Binding of [^{125}I]human FSH (*left panel*) or [^{125}I]hCG (*right panel*) to ovarian membranes from rats fed a control (CD), regular (RD), or EFA-deficient (EFA-D) diet measured at 10 and 20 days of age (FSH receptors) or 30 and 35 days of age (hCG receptors). *Left panel*, *, $P < 0.01$ *vs.* RD; **, $P < 0.001$ *vs.* CD and RD. *Right panel*, *, $P < 0.025$ *vs.* CD and RD; **, $P < 0.002$ *vs.* RD.

are low, the FSH receptor content of deficient animals was reduced ($P < 0.01$) only when compared to that of rats fed the regular diet. Alterations in hCG (LH) receptors assessed in late juvenile-peripubertal rats were less pronounced than those in FSH receptors (Fig. 6, *right panel*). However, at both 30 and 35 days of age EFA-deficient rats had a decreased ($P < 0.025$) content of hCG (LH) receptors.

Ovarian steroidal response to gonadotropins

To determine the physiological significance of reduced ovarian hCG receptor binding in juvenile EFA-deficient rats, the release of both E_2 and P from ovaries of 30- to 33-day-old EFA-deficient or rats fed the regular diet was determined *in vitro* after the addition of hCG to the incubation medium. hCG-stimulated E_2 release in ovaries from rats fed the regular diet was highly variable, presumably because of different degrees of follicular maturation at this juvenile-peripubertal transitional period (Fig. 7, *right panel*). The response of EFA-deficient rats was lower ($P < 0.05$) and less variable. Basal levels of E_2 release were also reduced in deficient rats. In contrast, there was no significant difference in ovarian P release between the two groups (Fig. 7, *left panel*).

Discussion

The present results demonstrate that deficiency of EFA initiated before fertilization does not affect the progression of pregnancy or the delivery of a healthy litter, but significantly delays the onset of reproductive capacity in the female offspring.

EFA deficiency, biochemically assessed in late juvenile rats born to EFA-deficient mothers, resulted in a striking depletion of serum n-6 fatty acids, among which linoleic acid (18:2 n-6) and arachidonic acid (20:4 n-6) were reduced to less than 10% of their normal values. In agreement with previous findings (8, 14, 15) a deficiency in n-3 fatty acids, albeit evident, was much less noticeable, with total n-3 fatty acid levels in serum decreased by only 50%. Most of the overt pathologies observed in classical long term EFA deficiency are attributed to a lack of n-6 fatty acids, since the signs are completely reversed by dietary n-6 fatty acid replacement (5, 6, 8). Dietary deprivation of n-3 fatty acids alone does not lead to decreased fertility, retarded growth or dermatitis, even when the deficiency is maintained for two or three generations (30, 31). Thus, n-3 fatty acid deficiency fails to affect reproductive processes, despite the fact that docosahexaenoic acid (22:6 n-3) is a major component of cell membrane phospholipids in the brain and gonads (32). The effects detailed here are, therefore, considered to result from a deficiency of n-6 fatty acids.

The delayed reproductive development observed in rats fed an EFA-deficient diet appears to stem from alterations in both hypothalamic and ovarian function. Pituitary dysfunction, although not explored, must also be considered as a potential contributing factor. Both

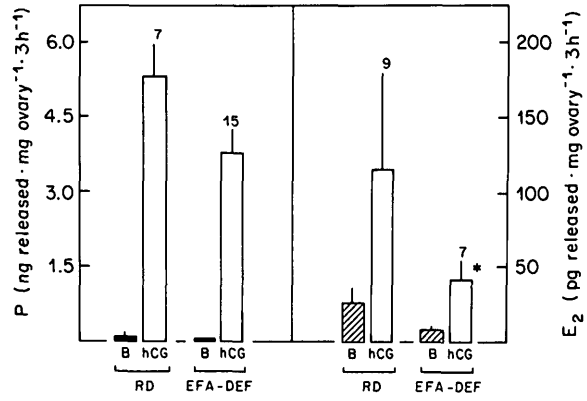

FIG. 7. *In vitro* release of P (*left panel*) and E_2 (*right panel*) from ovaries of 30- to 33 day-old rats fed a regular (RD) or EFA-deficient (EFA-D) diet. *, $P < 0.001$ *vs.* RD (by Mann-Whitney U test). B = basal release.

the hypothalamus and the ovaries exhibited a marked reduction in their ability to release PGE_2, a cyclooxygenase metabolite of arachidonic acid which has been implicated in the process of LHRH release (10, 12) and ovulation (11, 13). The observed decrease in PGE_2 production cannot be attributed to a decrease in the activity of either the PG synthase complex or of the other enzymes that catalyze the further metabolism of arachidonic acid to PGs and thromboxanes (9). Incubation of hypothalamic homogenates with exogenous labeled arachidonic acid resulted in a normal profile of metabolite formation, thus verifying the prediction based on the observed depletion in serum linoleic and arachidonic acids, that the reduced PGE_2 response to neurotransmitter or hormonal stimuli is caused by arachidonate deficiency.

The depressed hypothalamic PGE_2 response to NE is in all likelihood an important factor contributing to the lack of LH response to E_2 stimulation observed in EFA-deficient juvenile rats. The preovulatory LH surge induced by E_2 in prepubertal rats is associated with an increased capacity of NE to induce PGE_2 formation in the ME and an increased LHRH response to PGE_2 (33). During normal sexual development the LH surge in response to E_2 occurs earlier (within 30 h) in more mature than in younger animals, which require 54 h to respond (2). Since EFA-deficient rats failed to release LH on the day after E_2 administration, but eventually reached puberty, it must be concluded that EFA deficiency delayed the maturation of the central component of E_2 positive feedback.

An additional factor that probably contributed to the delayed hypothalamic maturation is a depressed production of E_2. Although serum E_2 levels were not measured, a reduction in steroid secretion is strongly suggested by the lower uterine weight and blunted ovarian E_2 response to hCG observed in EFA-deficient rats. It is known that E_2 can accelerate the maturation of the central component of E_2 positive feedback (1), presumably by promoting the accumulation of LHRH in the hypothalamus (34) and facilitating the release of LHRH in response to secretagogues (33, 35). In fact, ovariectomy of early juvenile animals has been shown to almost completely obliterate the LHRH response to PGE_2, a known LHRH secretagog, during the peripubertal phase of development (33). That ovarian development was compromised by the EFA deficiency was further demonstrated by the reduction in FSH and hCG/LH receptor number and decreased ovarian weight observed in EFA-deficient rats. The production of inhibin may have also been reduced, as suggested by the elevated FSH secretory rate found in the deficient animals.

The secretory output of PRL and GH was statistically similar in control and EFA-deficient rats. The apparent lack of change in PRL secretory output may have been related to the large variability observed in the control group. Indeed, mean PRL levels were generally low in EFA-deficient animals during development. This may have contributed to the ovarian dysfunction, since PRL has been shown to facilitate ovarian development (19). Interestingly, in spite of decreased ovarian secretory capacity mean serum LH levels were normal, a finding that may be related to the diminishing ability of the hypothalamic-pituitary unit of EFA-deficient rats to release LH in response to changes in steroid feedback control.

A surprising finding was that animals fed the control diet had delayed puberty compared to rats fed the regular diet. We have no explanation for this other than to suspect that the semipurified control diet lacked some trace mineral or yet to be defined essential components or that the two diets differ in palatability. The latter possibility is unlikely, however, as no differences in body weight between the two groups were detected.

The lower rate of body weight gain observed in EFA-deficient rats raises the question of whether the delayed puberty was due to the EFA deficiency itself or the mild undernutrition that may have resulted from a reduced food intake. It is known that chronic mild undernutrition can delay female puberty (for a review, see Ref. 36). It is doubtful, however, that food intake was lower in EFA-deficient rats compared with animals fed the control diet. With the exception of the source of fat, both diets have an identical composition. To assess the impact of a possible lower food intake by EFA-deficient rats we could have restricted food intake to a group of animals to obtain the same degree of weight gain as EFA-deficient rats. This, however, does not provide an adequate control, because EFA-deficient rats were born with a body weight already lower than control litters. Moreover, in earlier experiments in which litter size was adjusted to decrease the rate of body growth, the underfed animals exhibited VO at the same age as rats grown in normal size litters, but at a significantly lower body weight (37). Thus, although we cannot entirely rule out a contribution of mild undernutrition to the delayed puberty in EFA-deficient rats, the above considerations indicate that the main factor responsible for the delay is, indeed, the EFA deficiency. In addition, 1) puberty in the rat can be attained at different body weights (37, 38). Results of recent experiments further illustrate this concept. In these experiments, normally fed rats were found to have a rate of body growth similar to that of EFA-deficient rats, but did not shown delayed puberty (Lara, H. E., J. K. McDonald, and S. R. Ojeda, submitted). In these animals VO occurred at 34.7 ± 0.8 days and at a body weight of 111 ± 4 g. 2) Alterations in circulating FSH levels and ovarian content of gonadotropin receptors

were already observed in infantile EFA-deficient animals. At this time, the growth of the pups is entirely dependent on their milk intake. 3) Very importantly, underfeeding does not alter the central component of E_2 positive feedback (37, 39) and does not increase basal levels of plasma FSH (37). This is in marked contrast with the effects of EFA deficiency found in the present experiments.

In spite of the striking loss of n-6 fatty acids, all EFA-deficient rats eventually succeeded in reaching puberty. This suggests that either alternative mechanisms not involving arachidonic acid metabolites come into play, or that the response of target cells to decreased arachidonate-mediated stimulation is compensatorily enhanced. Congruent with our findings, EFA deficiency has also been shown to delay, but not to prevent, the biochemical development of rat forebrain (40). Although EFA do not appear essential for general membrane function (41), arachidonic acid has been shown to exert differential effects on distinct neurotransmitter systems in the brain; for instance, arachidonic acid elicits decreases in ion channel function (42) and reuptake mechanisms (43) for inhibitory and excitatory amino acids, respectively. Thus, a deficiency in arachidonic acid would differentially amplify the action of inhibitory *vs.* excitatory amino acid systems, an effect that could conceivably delay the acquisition of full reproductive capacity at any level of the hypothalamic-pituitary-gonadal axis.

Although the underlying mechanism remains to be established definitively, the present results demonstrate that availability of n-6 essential fatty acids is necessary for the timely activation of the hypothalamic-pituitary-ovarian axis that leads to the acquisition of reproductive competence.

Acknowledgments

We are grateful to Dr. G. D. Niswender (Colorado State University) for the supply of antisera for E_2, P, and LH RIAs. Purified LH for radioiodination was kindly provided by Dr. L. E. Reichert (Albany School of Medicine, Albany, NY). The antiserum for the PGE_2 RIA was generously provided by Dr. W. B. Campbell (Department of Pharmacology, University of Texas Health Sciences Center, Dallas). Highly purified hCG for iodination was a gift from Dr. R. E. Canfield (Columbia University College of Physicians and Surgeons, New York, NY). Purified FSH was obtained from the NIDDK-NIH Pituitary Hormone Program. We are also grateful to Dr. W. B. Campbell (Dallas, TX) for his assistance in the HPLC analysis of cyclooxygenase products. We thank Ms. Janie Gliessman for typing the manuscript.

References

1. Caligaris L, Astrada JJ, Taleisnik S 1972 Influence of age on the release of luteinizing hormone induced by oestrogen and progesterone in immature rats. J Endocrinol 55:97
2. Andrews WW, Mizejewski GJ, Ojeda SR 1981 Development of estradiol positive feedback on LH release in the female rat: a quantitative study. Endocrinology 109:1401
3. Advis JP, Andrews WW, Ojeda SR 1979 Changes in ovarian steroidal and prostaglandin E responsiveness to gonadotropins during the onset of puberty in the female rat. Endocrinology 104:653
4. Burr GO, Burr MM 1930 On the nature and role of the fatty acids essential nutrition. J Biol Chem 86:587
5. Aaes-Jorgensen E 1961 Essential fatty acids. Physiol Rev 41:1
6. Holman RT 1968 Essential fatty acid deficiency. Prog Chem Fat 9:275
7. O'Brien JS, Sampson EL 1965 Fatty acid and fatty aldehyde composition of the major brain lipids in normal human gray matter, white matter and myelin. J Lipid Res 6:545
8. Neuringer M, Anderson GJ, Connor WE 1988 The essentiality of n-3 fatty acids for the development and function of the retina and brain. Annu Rev Nutr 8:517
9. Needleman P, Turk J, Jakachik BA, Morrison AR, Lefkowith JB 1986 Arachidonic acid metabolism. Annu Rev Physiol 55:69
10. Ojeda SR, Urbanski HF, Junier M-P, Capdevila J 1989 The role of arachidonic acid and its metabolites in the release of neuropeptides. Ann NY Aca Sci 559:192
11. Behrman HR 1979 Prostaglandins in hypothalamo-pituitary and ovarian function. Annu Rev Physiol 41:685
12. Ojeda SR, Urbanski HF 1988 Intracellular regulatory mechanisms of LHRH secretion and the onset of female puberty. In: Imura H (ed) Neuroendocrine Control of the Hypothalamic-Pituitary System. Japan Scientific Society Press, Tokyo, p 49
13. LeMaire WJ, Koos RD, Clark MR, Curry TE, Holmes PV, Janson PO, Sogn JH, Ahren KEB 1985 Studies on the role of prostaglandins in ovulation using the isolated perfused ovary as a model. In: Hayaishi O, Yamamoto S (eds) Advances in Prostaglandin, Thromboxane and Leukotriene Research. Raven Press, New York, p 589
14. Alling C, Bruce A, Karlsson I, Svennerholm J 1974 The effect of different dietary levels of essential fatty acids on lipids of rat cerebrum during maturation. J Neurochem 23:1262
15. Paoletti R, Galli C 1972 Effects of essential fatty acid deficiency on the central nervous system in the growing rat. In: Elliot K, Knight J (eds) Lipids, Malnutrition and the Developing Brain. Ciba Found Symp, Elsevier, Amsterdam, p 121
16. Bligh EG, Dyer WJ 1959 A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911
17. Rose HG, Oklander M 1965 Improved procedure for the extraction of lipids from human erythrocytes. J Lipid Res 6:428
18. Morrison WR, Smith LM 1965 Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. J Lipid Res 6:428
19. Advis JP, Ojeda SR 1978 Hyperprolactinemia-induced precocious puberty in the female rat: ovarian site of action. Endocrinology 103:924
20. Negro-Vilar A, Ojeda SR, McCann SM 1979 Catecholaminergic modulation of lutenizing hormone-releasing hormone release by median eminence terminals in vitro. Endocrinology 104:1749
21. Ojeda SR, Negro-Vilar A, McCann SM 1979 Release of prostaglandin E (PGEs) by hypothalamic tissue: evidence for their involvement in catecholamine-induced LHRH release. Endocrinology 104:617
22. Legan SJ, Coon GA, Karsch FJ 1975 Role of estrogen as initiator of daily LH surges in the ovariectomized rat. Endocrinology 96:50
23. Harms PG, Ojeda SR 1974 A rapid and simple procedure for chronic cannulation of the rat jugular vein. J Appl Physiol 36:391
24. Niswender GD, Midgley AR, Monroe SE, Reichert LE 1968 Radioimmunoassay for rat luteinizing hormone with antiovine LH serum and ovine LH-I^{131}. Proc Soc Exp Biol Med 128:807
25. Campbell WB, Gomez-Sanchez CE, Adams BV 1980 Role of prostaglandins in angiotensin-induced steroidogenesis. Hypertension 2:471
26. Ojeda SR, Campbell WB 1982 An increase in hypothalamic capacity to synthesize prostaglandin E_2 precedes the first preovulatory surge of gonadotropins. Endocrinology 111:1031
27. Smith S, Ojeda SR 1981 Changes in ovarian luteinizing hormone and follicle-stimulating hormone receptor content and in gonadotropin-induced ornithine decarboxylase activity during prepubertal

and pubertal development of the female rat. Endocrinology 109:152
28. Richards JS, Ireland JJ, Rao M, Bermeath GA, Midgely Jr AR, Reichert LE 1976 Ovarian follicular development in the rat: hormone receptor regulation by estradiol, follicle stimulating hormone and luteinizing hormone. Endocrinology 99:1562
29. Burton K 1956 A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J 62:315
30. Lamptey MS, Walker BL 1976 A possible essential role for dietary linolenic acid in the development of the young rat. J Nutr 106:86
31. Tinoco J, Williams MA, Hincebergs I, Lyman RL 1971 Evidence for non-essentiality of linolenic acid in the diet of the rat. J Nutr 101:937
32. Tinoco J 1982 Dietary requirements and functions of alpha-linolenic acid in animals. Prog Lipid Res 21:1
33. Ojeda SR, Urbanski HF, Katz KH, Costa ME 1986 The activation of estradiol positive feedback at puberty: estradiol sensitizes the LHRH releasing system at two different biochemical steps. Neuroendocrinology 43:259
34. Kalra SP 1976 Tissue levels of luteinizing hormone-releasing hormone in the preoptic area and hypothalamus, and serum concentrations of gonadotropin following anterior hypothalamic deafferentation and estrogen treatment of the female rat. Endocrinology 99:101
35. Drouva SV, Laplante E, Gantron JP, Kordon C 1984 Effects of 17β-estradiol on LHRH release from rat mediobasal hypothalamic slices. Neuroendocrinology 38:152
36. Bronson FH, Rissman EF 1986 The biology of puberty. Biol Rev 61:157
37. Ronnekleiv OK, Ojeda SR, McCann SM 1978 Undernutrition, puberty and the development of estrogen positive feedback in the female rat. Biol Reprod 19:414
38. Wilen R, Naftolin F 1977 Pubertal food intake, body length, weight and composition in the well-fed female rat. Pediatr Res 11:701
39. Foster DL, Olster DH 1985 Effect of restricted nutrition on puberty in the lamb: patterns of tonic luteinizing hormone (LH) secretion and competency of the LH surge system. Endocrinology 116:375
40. Karlsson I, Svennerholm L 1978 Biochemical development of rat forebrains in severe protein and essential fatty acid deficiencies. J Neurochem 31:657
41. Lee AG, East JM, Froud RJ 1986 Are essential fatty acids essential for membrane function? Prog Lipid Res 25:41
42. Schwartz RD, Skolnick P, Paul SM 1988 Regulation of gamma-aminobutyric acid/barbiturate receptor-gated chloride ion flux in brain vesicles by phospholipase A2: possible role of oxygen radicals. J Neurochem 50:565
43. Yu ACH, Chan PH, Fishman RA 1987 Arachidonic acid inhibits uptake of glutamate and glutamine but not of GABA in cultured cerebellar granule cells. J Neurosci Res 17:424