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Alteration of Gonadotropin-releasing Hormone and Luteinizing Hormone β-Subunit mRNA Levels in Neonatally Estrogenized Female Rats

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Treatment of newborn female rats with gonadal steroids induces permanent sterility in adulthood. We investigated the alteration in expression patterns of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) in neonatally estrogenized sterile rats (ESR). Newborn female rats received daily injections of 17β-estradiol (E, 10 μg) from the day of birth (day 1) to postnatal day 5. Controls were subjected to vehicles over the same period. All animals were sacrificed on week 7 after birth. Hypothalamic GnRH mRNA levels were markedly higher in all ESR than in controls, while hypothalamic GnRH contents in ESR increased in proportion to the frequency of daily administration of E. However, both pituitary LHβ mRNA and serum LH levels were inversely decreased by the same treatment. The data indicate that neonatal exposure of E equally elevates the expression of GnRH gene, but reduces the secretion of GnRH, accordingly leading to attenuation of LHβ gene expression and circulating LH levels. The temporal effect of E and/or progesterone (P) on GnRH and LHβ mRNA levels was also examined in ESR. Newborn female rats were daily injected with E (10 μg) or vehicle for five successive days from day 1 and ovariectomized at week 5. They were implanted with E (235 μg/ml) two days prior to week 7, injected with P (1 mg) 42 h later, and sacrificed 7 h after P administration. In ovariectomized controls, hypothalamic GnRH mRNA levels were dropped to half by treatment of E and restored by subsequent treatment of P. The negative feedback action of E on GnRH mRNA levels observed in ovariectomized rats was completely blocked by neonatal exposure of E. The change in pituitary LH mRNA levels was similar to that in hypothalamic GnRH mRNA levels. Taken together, the results suggest that neonatal treatment of E alters the synthesis and release of GnRH in adulthood and furthermore blocks the negative feedback regulation of E which occurs normally after ovariectomy.

Gonadal steroids play an essential role in the regulation of ovulation in cyclic female rats, that is characterized by a proestrous surge of gonadotropin (Brann and Mahesh, 1991). The event is prevented by the treatment of neonatal female rats with androgen or estrogen, causing permanent anovulatory sterility in adulthood (Barraclough, 1961; Gorski, 1963; Boverdig et al., 1972; Mennin et al., 1974; Aihara and Hayashi, 1989; Hayashi and Aihara, 1989; Hayashi et al., 1991; Pinilla et al., 1993). These animals show low levels of luteinizing hormone (LH) in both blood and pituitary (Hayashi and Aihara, 1989; Pinilla et al., 1993). Exogenous gonadotropin-releasing hormone (GnRH) is capable of inducing surge-like release of gonadotropins in steroid-sterilized female rats (Boverdig et al., 1972; Mennin et al., 1974), indicating that pituitary has normal function in response to GnRH. However, hypothalamic GnRH contents are elevated (Hayashi et al., 1991) and electric stimulation of the medial preoptic area evokes less LH release in steroid-treated rats than in normal proestrous animals (Chappel and Barraclough, 1976). Thus the sterility observed may be due not only to the modification of releasing mechanism of the GnRH neuronal system but also to the lack of neural signal manipulating the discharge of GnRH.

During development, GnRH neurons migrate from the olfactory placode and reside in the preoptic area and this process is completed around birth (Schwanzel-

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Fukuda et al., 1985; Wray and Hoffman, 1986; Schwanzel-Fukuda and Pfaff, 1989). Under the influence of neonatal steroids, the sexually dimorphic nucleus of the preoptic area and the anteroventral periventricular nucleus undergo volumetric changes (Jacobson et al., 1981; Davis et al., 1995; Davis et al., 1996). These areas appear to be involved in the neural circuit responsible for the synthesis of GnRH in female rats. Lesions of the area develop an anovulatory state by disruption of the phasic release of GnRH (Terasawa et al., 1980; Wiegand et al., 1980). The nuclei contain estrogen-concentrating cells and are immunoreactive for the estrogen receptor (Bloch et al., 1992; Herbison and Theodosis, 1992), indicating a putative action site of estrogen.

Gonadal steroids have been widely employed to examine their temporal effects on reproductive hormones in the absence of the ovaries. Ovariectomy of adult female rats induces apparent increases in LH, but decreases in hypothalamic GnRH contents and GnRH release into hypophysial portal vessel (DePaolo and Barraclough, 1979; Kalra and Kalra, 1989). The treatment of ovariectomized animals with estrogen reduces circulating LH, but subsequent treatment of progesterone (P) to ovariectomized estrogen treated rats induces surge-like release of GnRH and LH (Kim et al., 1989; Zanisi and Messi, 1991). In contrast, the similar hormonal manipulation fails to evoke a significant increase of LH in female rats that received estrogen in the early period of life (Aihara and Hayashi, 1989; Hayashi and Aihara, 1989; Hayashi et al., 1991). Thus, neonatal exposure of estrogen is likely to eliminate the negative feedback action of steroid on LH. It is, however, unknown whether the neonatal treatment of estrogen regulates the expression of GnRH and LH in female rats.

The present study was designed to examine the impact of neonatal 17β-estradiol (E) on the GnRH and LHβ gene expression in the estrogenized sterile rats (ESR) and to further examine the activational effect of E and/or P on the parameters in the ESR followed by ovariectomy.

Materials and Methods

Animals and Tissue Preparations

Sprague-Dawley rats were raised in the Seoul National University Animal Breeding Center. They were housed in a temperature-controlled room (23℃) under a 14 h light and 10 h dark photoperiod (light on at 06:00) with food and water *ad libitum*. Offsprings were weaned at week 3 and sacrificed by decapitation on the scheduled day. Trunk blood was collected and stored at 4℃ overnight for serum collection. Ovaries were excised and weighed. Pituitary and hypothalamus were excised on dry ice and stored frozen at -70℃. To determine GnRH contents, the hypothalamus was homogenized in 1 ml of 0.02 N acetic acid with a Dounce homogenizer on ice. Samples were spun at 10,000 g for 30 min at 4℃. Supernatant was transferred to new tubes and stored at -20℃.

Experimental design

The experimental scheme is diagramed in Fig. 1. One set of randomly selected newborn female rats was injected daily with E (10 μg, dissolved in sesame oil, in a volume of 20 μl), from the day of birth (day 1) to postnatal day 5. Depending on the number of neonatal treatments of E, five groups were assigned (Fig. 1A). Controls were subjected to vehicle for five consecutive days from day 1. All litters were separated from the mother at week 3 and sacrificed at the age of week 7. Hypothalamus, pituitary, ovary, and serum were prepared as mentioned above. Another set of animals was subjected to daily injections of E (10 μg) or vehicle for five consecutive days from day 1 (Fig. 1B). Animals were divided into two subgroups. One subgroup was ovariectomized at week 5 under light ether anesthesia. The other subgroup remained ovary intact. The ovariectomized animals were implanted with E (235 μg/ml) for 12 days and then received a single injection of P (1 mg) 42 h later. Six hours following P treatment, all animals were killed and hypothalamus, pituitary, ovary, and serum were prepared as mentioned above.

Total RNA Extraction

Total cytoplasmic RNA from hypothalamus and pituitary was extracted by acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Briefly, the hypothalamic fragments were homogenized in 600 μl denaturing solution containing 4 M guani

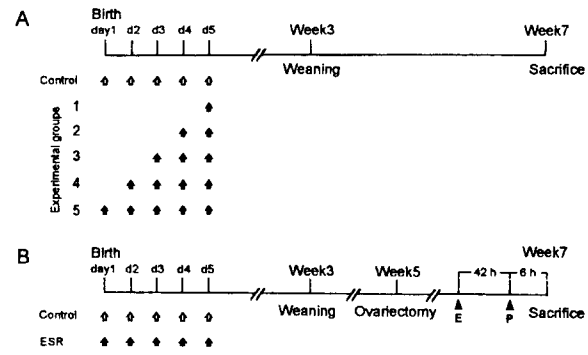

Fig. 1. Schematic diagrams of experiments. A, Newborn female rats were daily injected with E (10 μg) from the day of birth (day 1) to postnatal day 5. Five groups were assigned depending on the number of neonatal treatment of E. Controls received vehicle for five consecutive days from day 1. All litters were separated from the mother at week 3. They were sacrificed at week 7. B, Newborn female rats were daily injected with E (10 μg, ESR) or vehicle (Control) for 5 consecutive days from day 1. They were ovariectomized bilaterally at week 5 following weaning at week 3. They were allowed to recover for 12 days, implanted with E (235 μg/ml), and received a single injection of P (1 mg) 42 h later. Six hours following P treatment, animals were sacrificed.

dinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% N-lauryl sarcosine, and 0.1 M 2-mercaptoethanol. Sixty μl of 2 M sodium acetate (pH 4), 600 μl of water-saturated phenol, and 120 μl of chloroform-isoamyl alcohol mixture (49:1) were added. After cooling on ice for 15 min, the samples were centrifuged at 10,000 g at 4℃ for 20 min and precipitated with ethanol. After washing with 70% ethanol, the RNA pellet was dried under vacuum and dissolved in 20 μl of sterilized distilled water. RNA contents were then quantified at A_{260} absorbency. The optical density ratio of A_{260} to A_{280} ranged from 1.8 to 2.0.

Northern blot hybridization assay

Hypothalamic RNA (30 μg) and pituitary RNA (10 μg) dissolved in distilled water were denatured in 50% formamide, 6.2% formaldehyde, 20 mM MOPS (3-[N-morpholino] propanesulfonic acid), 5 mM sodium acetate, and 1 mM EDTA at 60℃ for 5 min. Electrophoresis was performed at 100 volts for 1.5 h in 1.2% agarose gel. After RNA was transferred to Nytran filter (pore size: 0.45 μm, Schleicher & Schuell) for 18 h by diffusion blotting, the filter was dried and baked at 80℃ for 2 h. The rat GnRH cDNA clone inserted into plasmid pGEM4, which was generously provided by Dr. Kelly Mayo (Northwestern University, U.S.A.), was used as a template DNA for an *in vitro* synthesis of RNA probe. The construct was linearized and RNA probe labeled with ^{32}P-UTP (New England Nuclear) to a high specific activity (1.3×10^9 cpm/g) was transcribed using SP6 RNA polymerase (15 units/μl, BRL) (Blum, 1989). The LHβ cDNA probe and 18S cDNA for the internal control probe were labeled with ^{32}P-dCTP (New England Nuclear) by the random hexamer extension method (Feinberg and Vogelstein, 1984). The labeled probes were separated from unincorporated ^{32}P-dCTP by a Sephadex G-50 column. Prehybridization was carried out at 42℃ for 2 h in a heat-sealable plastic bag (Kapak) with hybridization buffer consisting of 50% deionized formamide, 5 x SSPE, 5 x Denhardt's solution (1 x Denhardt's solution: 0.02% polyvinylpyrrolidone, 0.02% ficoll, and 0.02% BSA), 0.1% SDS, and 2 mg of heat-denatured salmon sperm DNA. After addition of ^{32}P-labeled RNA probe hybridization was performed for 20 h at 62℃ and 42℃ for GnRH and LHβ, respectively. The Nytran membrane was washed twice with 2 x SSC and 0.1% SDS at room temperature for 20 min, followed by the second washing with 0.07 x SSC, 0.5% SDS, and 5 mM EDTA (pH 8) at 62℃ for 5 min. The membrane was then dried and exposed to X-ray film (Fuji) at -70℃ for 2 days. The same membrane was then rehybridized with an 18S DNA probe under the same conditions except for the hybridization temperature of 42℃. The rehybridized membrane was washed twice with 2 x SSC and 0.1% SDS at room temperature for 20 min, followed by a washing 0.1 x SSC and 0.1% SDS at 42℃ for 20 min.

Radioimmunoassay (RIA) for GnRH and LH

Hypothalamic GnRH content was determined by GnRH RIA using the Chen-Ramirez GnRH antiserum (CRR-11-B-72) at a final dilution of 1 : 200,000. Synthetic GnRH (Sigma) was used for iodination and reference standard. The sensitivity at 80% binding was approximately 0.5 pg/tube. The intra- and inter-assay coefficient of variations was 4-5% and 5-10% for 2 pg dose of synthetic GnRH, respectively.

Serum LH levels were measured by double antibody RIA using reagents kindly provided by the National Pituitary Agency. The tracer NIADDK-rLH-I9 was iodinated by the chloramine-T method. The antiserum was NIADDK-rLH-S-10 and the reference preparation was NIADDK-rLH-RP-2. LH levels were expressed as NIADDK RP2 units. Intra- and inter-assay Coefficient variations were 7.2 and 11.2%, respectively.

Data analysis

The amount of RNA applied to the gel was determined by rehybridization with ^{32}P-labeled 18S DNA control probe when appropriate. The hybridization signals were determined by scanning the appropriately exposed autoradiogram with densitometry. Hypothalamic GnRH and pituitary LH mRNA levels were expressed as a percentage of the control value. The data for GnRH contents, serum LH concentrations, and ovarian weights were statistically evaluated using one-way analysis of variance followed by Fisher's least significance difference test for a *post-hoc* comparison. The level of significance was set at $P<0.05$.

Results

Fig. 2 shows the hypothalamic GnRH contents, serum LH levels, and ovarian weights in control and ESR. There was a gradual increase of hypothalamic GnRH contents in proportion to the numbers of daily E injections to the perinatal female rats. The amount of GnRH was significantly elevated by a single injection of E on day 5 (day 1 represents the day of birth), compared to controls ($p<0.05$). More frequent treatments of E correlated with higher levels of GnRH contents and the highest levels of GnRH contents were observed by daily treatments of E for five successive days from day 1. In contrast, serum LH levels were gradually reduced by the numbers of daily E injections. The highest levels were shown in controls and more frequent treatments of E correlated with lower levels of serum LH. Ovarian weights were significantly lower in the animals that received daily E injections for 4 and 5 days, compared to the other groups ($p<0.05$).

Hypothalamic GnRH mRNA levels in all ESR were significantly higher than those in controls ($p<0.05$,

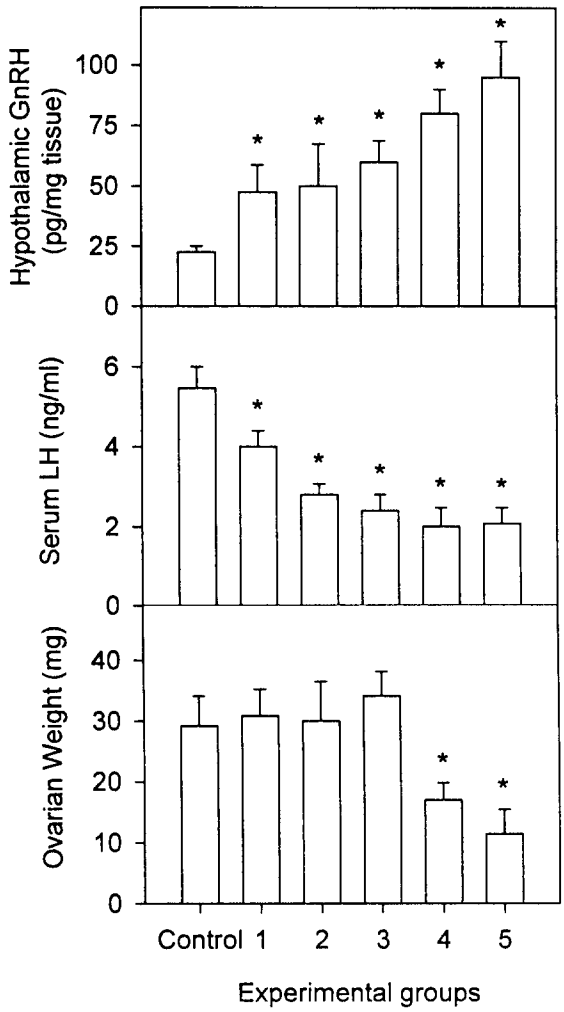

Fig. 2. Influence of neonatal treatment of E on hypothalamic GnRH contents, serum LH, and ovarian weights. The injection schedule is as described in Fig. 1A. Values are mean SEM (n=6-10). * indicates significant differences compared to control (P<0.05).

Fig. 3). Even a single injection of E on day 5 profoundly increased GnRH mRNA levels. However, GnRH mRNA levels did not mirror the GnRH contents shown in Fig. 2.

Pituitary LHβ mRNA levels were shown in Fig. 4. The level of LHβ mRNA was highest in controls and declined with the numbers of the neonatal treatment of E. The gradual decrement paralleled the serum LH values and was conversely related to the hypothalamic GnRH contents (compare Fig. 4 and Fig. 2).

Since it has been well established that P induces gonadotropin surge in E-primed ovariectomized adult rats, the influence of neonatal treatment of E on the expression of GnRH and LHβ mRNA levels was examined in the same experimental paradigm. In order to ensure the results observed in Fig. 2, some animals were sacrificed at week 7 following

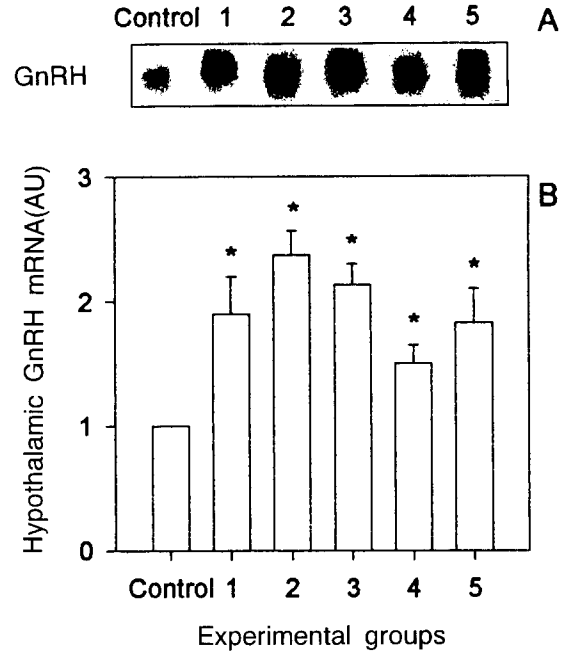

Fig. 3. Effect of neonatal treatment of E on hypothalamic GnRH mRNA levels in ESR. A, A representative autoradiogram of Northern blot analysis. B, The quantification of GnRH mRNA levels as a percentage of control levels. The injection schedule is as described in Fig. 1A. Values are mean SEM (n=3). * indicates significant differences compared to control (P<0.05).

treatment of vehicle or daily injections of E for five consecutive days from day 1. Hypothalamic GnRH contents were significantly greater in ESR than in control (Fig. 5). In contrast, serum LH levels and ovarian weights were markedly decreased in ESR. The results are the same as shown in Fig. 2.

Fig. 6 shows the effects of E and/or P on hypothalamic GnRH mRNA levels in control and ESR followed by ovariectomy. The E-priming to control rats resulted in a conspicuous decrease of hypothalamic GnRH mRNA levels, which was restored by subsequent treatment of P. However, P alone did not affect hypothalamic GnRH mRNA levels. In ESR, the effect of E on GnRH mRNA levels shown in controls was blocked. The administration of E and/or P did not alter GnRH mRNA contents in ESR. There was no difference in GnRH mRNA levels between control and ESR except for the E-primed group.

Alterations in the pituitary LHβ mRNA levels were similar to those in the hypothalamic GnRH mRNA levels (Figs. 6 and 7). The E-priming to the ovariectomized control rats decreased LHβ mRNA levels to nearly half and it was recovered by subsequent treatment of P. However, P alone had no effect. In ESR, there were no alterations in LHβ mRNA contents by E and/or P treatment. The reduction of LHβ mRNA contents by E in control rats were blocked by the neonatal exposure of E.

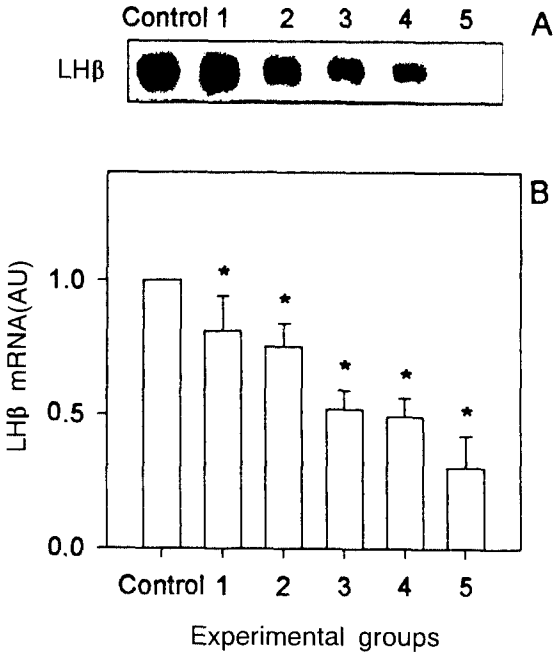

Fig. 4. Effect of neonatal treatment of E on pituitary LHβ mRNA levels in ESR. A, A representative autoradiogram of Northern blot analysis. B, The quantification of LHβ mRNA levels as a percentage of control levels. The injection schedule is as described in figure 1A. Values are mean SEM (n=3). * indicates significant differences compared to control (P<0.05).

Discussion

The present results provide direct evidence that E induces an increment of GnRH gene expression and alters the releasing mechanism of GnRH in adulthood when administered daily to the newborn female rats up to day 5. Moreover, neonatal treatment of E to ovariectomized ESR also eliminates the negative feedback effects of E on both hypothalamic GnRH mRNA and pituitary LHβ mRNA levels in adulthood.

Earlier studies showed that exogenous treatment with steroids early in life irreversibly modifies the brain of adult rats. Castration shortly after birth leads to reduction of the volume of sexual dimorphic nucleus of the preoptic area (Davis et al., 1995), which is restored to the level of intact male rats by the replacement of testosterone immediately after orchidectomy (Jacobson et al., 1981). The nuclei of the steroid-treated neonatal females are larger than those of non-treated females, but smaller than those of the intact males (Jacobson et al., 1981). Since the dimensions of the sexual dimorphic nucleus are larger in male rats than in female rats *per se*, it is apparent that neonatal steroids promote volumetric changes of the specific brain areas. E appears an active substance because of the presence of the enzyme aromatase that transforms testosterone into E, which occurs in the central nervous system during the fetal or neonatal life (McEwen et al., 1977). Accordingly, the ESR is

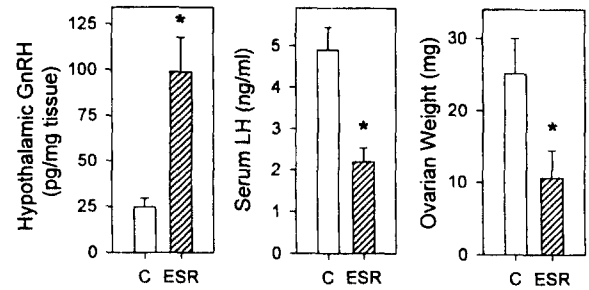

Fig. 5. Influence of neonatal treatment of E on hypothalamic GnRH contents, serum LH, and ovarian weights. The injection schedule is as described in Fig. 1B. Control is expressed as C on the abscissa. Values are mean SEM (n=6-10). * indicates significant differences compared to control (P<0.05).

considered to be a reliable model to examine the organizational effect of steroids.

In the present study, hypothalamic GnRH mRNA levels were uniformly augmented in ESR regardless of the numbers of daily injections of E. In normal cycling female rats, GnRH mRNA levels vary along with the estrous cycle (Zoeller and Young III, 1988; Gore and Roberts, 1995). Particularly, on the diestrus, GnRH mRNA levels are sustained high and followed by a decrease in the morning of the proestrus. In the afternoon of the proestrus, GnRH mRNA levels increase when the circulating E levels drop precipitously (Zoeller and Young III, 1988). Thus, the increased expression of GnRH gene found in ESR is comparable to the diestrous stage of the normal adult rat. Both animals show low levels of plasma LH and high levels of hypothalamic GnRH mRNA and GnRH contents (Hayashi et al., 1991; Pinilla et al., 1993). They also display a similar pattern of the numbers of GnRH neurons and their axonal projections to the median eminence (King et al., 1980). No distinct differences were found in the topographical distribution of the E receptor-positive neurons in the hypothalamus as adults. Thus, the sterility induced by neonatal treatment of E can be attributed to the functional alteration of GnRH neurons, but not to the structural change, in spite of the changes in volume of the sexually dimorphic nucleus of the preoptic area (Jacobson et al., 1981; Davis et al., 1995; Davis et al., 1996). Under the discrete condition, diestrous-stage rats have a potential to ovulate through the surge of gonadotropins, whereas ESR are unable to do so. This discrepancy may be due to the steroid milieu. Cyclic females undergo fluctuation of E and P secreted from the ovary, but the ESR show constantly high levels of E (Castro-Vazquez and McCann, 1975). In ESR, the constant high levels of E may cause the alteration of regulatory function of ovarian steroids, which is probably fulfilled by neonatal treatment of E during the development of GnRH neuronal system. The GnRH-immunoreactive neurons that originate from the medial olfactory pit migrate and reside in the preoptic area around birth (Schwanzel-

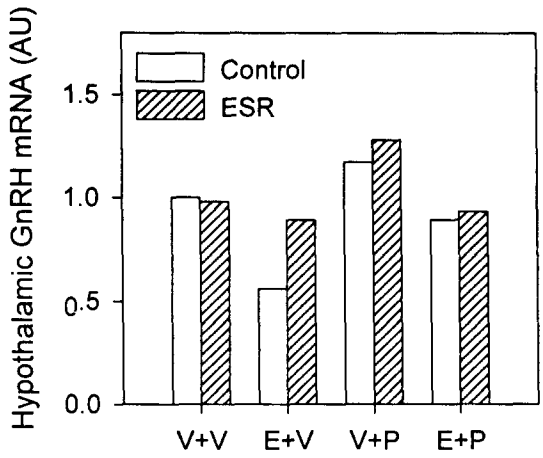

Fig. 6. Effects of E and/or P on hypothalamic GnRH mRNA levels in ovariectomized control and ESR rats that received vehicle (V) or E for five consecutive days from day 1. The injection schedule is as described in Fig. 1B. Following ovariectomy, animals were implanted with V or E and 42 h later, received a single injection of V or P, expressed by V+V, E+V, V+P, and E+P.

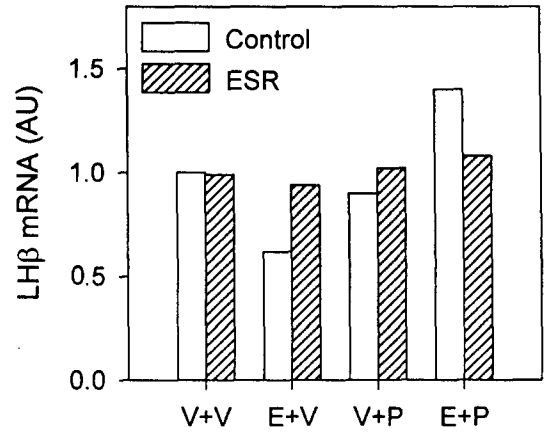

Fig. 7. Effects of E and/or P on Northern blot analysis of the pituitary LHβ mRNA in ovariectomized control and ESR rats that received vehicle (V) or E for five consecutive days from day 1. The injection schedule is as described in Fig. 1B. Following ovariectomy, animals were implanted with V or E and 42 h later, received a single injection of V or P.

Fukuda et al., 1985; Schwanzel-Fukuda and Pfaff, 1989). The adult-like GnRH perikarya and fibers are formed perinatally (Chappel and Barraclough, 1976; Schwanzel-Fukuda et al., 1985; Wray and Hoffman, 1986). Therefore, it appears that neonatal treatment of E can modify the organization of GnRH-generating system. This is supported by the previous finding that pituitary and plasma LH levels were remarkably suppressed during postnatal day 5 to 15 right after E treatment on day 1 (Pinilla et al., 1993). The present study demonstrates that gene expression of GnRH in adulthood is influenced by neonatal exposure of E.

Pituitary LHβ mRNA levels were suppressed in proportion to the numbers of daily injections of E until postnatal day 5. LHβ mRNA levels are normally depressed on diestrus following estrus (Zmeili et al., 1986). Similar results were reported in middle-aged persistent estrus animals showing low levels of LHβ mRNA expression (Matt et al., 1993). Circulating E levels are low in diestrus, but chronically high in ESR. Thus, ESR appear to possess an unusual physiological function compared to the normal cyclic females. This suggests that the reduced level of LHβ mRNA may be attributed to the reduced secretion of GnRH from the hypothalamus.

It has been well known that serum LH reflects hypothalamic GnRH contents. The increase in hypothalamic GnRH contents and the decrease in serum LH levels detected in ESR sacrificed at week 7 are in accordance with the previous findings that the releasing mechanism of GnRH is suppressed after neonatal treatment of E (Hayashi and Aihara, 1989; Hayashi et al., 1991). However, it has also been reported that both plasma LH and hypothalamic GnRH contents are not affected by the neonatal treatment of E at 90 and 100 days of age (Hayashi et al., 1991; Pinilla et al., 1993). In ESR followed by prepubertal ovariectomy, plasma LH levels are much lower at 50 days than at 100 days of age (Aihara and Hayashi, 1989; Hayashi and Aihara, 1989; Hayashi et al., 1991). When the regulatory action of steroids on LH secretion was evaluated in ovariectomized ESR, E had no effect in reducing plasma LH at both ages (Aihara and Hayashi, 1989; Hayashi and Aihara, 1989; Hayashi et al., 1991). In contrast, the augmented plasma LH levels by P after E-priming were noticed at 100 days, but not at 50 days of age (Aihara and Hayashi, 1989). Accordingly, it appears that the impact of neonatal E on the reproductive activity could be dependent on the period of time during which the animals are kept following neonatal treatment of E. There is evidence that at around 50 days of age, ESR demonstrates damaged secretory system of tropic hormones. On the other hand, the unexpected elevation of hypothalamic GnRH mRNA levels observed in ESR suggests that the transcription of GnRH gene may be separable from the secretion machinery.

The ovarian weights were significantly reduced in ESR given consecutive injections of E for 4 and 5 days. The other treatments were incapable of inducing ovarian atrophy. Although a single injection of E at postnatal day 5 was enough to increase hypothalamic GnRH mRNA levels and GnRH contents, and suppressed pituitary LHβ mRNA and serum LH levels, it was insufficient to exert its action on the ovarian mass. The reason is unclear, since the present study did not measure follicle-stimulating hormone levels. In a previous report, a single injection of E on day 1 induced ovarian atrophy (Pinilla et al., 1993). Thus, neonatal E-induced ovarian involution is seemingly restricted to at least postnatal day 1 and 2 in this experiment.

The activational effect of steroids on the GnRH mRNA levels was further examined in ovariectomized ESR. The effect of E and/or P on hypothalamic GnRH mRNA levels as well as circulating LH levels in ovariectomized adult rats has well been established (Zoeller et al., 1988; Cho et al., 1994; Peterson et al., 1995). In contrast, the same hormonal treatment did not alter plasma LH levels (Aihara and Hayashi, 1989; Hayashi and Aihara, 1989), but reduced hypothalamic GnRH contents at day 50 of ESR after ovariectomy (Hayashi et al., 1991). In the present study, hypothalamic GnRH mRNA levels in ovariectomized control rats were markedly suppressed by E treatment and restored by subsequent treatment of P. These results substantiate that GnRH gene expression in adult is indeed inhibited by E priming in the absence of ovary, as described previously (Castro-Vazquez and McCann, 1975; Gore and Roberts, 1995). In the ESR, hypothalamic GnRH mRNA levels were not affected by temporal treatment of E and/or P. On the other hand, it has previously been reported that GnRH mRNA levels were lowered by temporal E in the androgen-sterilized ovariectomized rats (Zoeller et al., 1988), which is different from our results. The contradictory data may be due to the period during which animals were housed following neonatal treatment of E. In the present experiment, ESR was sacrificed at week 7, but androgen-sterilized rats were killed at around 100 days of age (Zoeller et al., 1988). The additional period of time may allow the animals to recover from the impact of neonatal exposure of E, as shown by plasma LH that is significantly increased by P under pretreatment of E following prepubertal ovariectomy (Aihara and Hayashi, 1989; Hayashi and Aihara, 1989).

Similarly to the GnRH mRNA levels, the pituitary LHβ mRNA levels were also changed by temporal treatment of E and/or P following ovariectomy, which agrees with earlier reports (Gharib et al., 1986; Zmeili et al., 1986; Shupnik et al., 1988; Brann et al., 1993). In ESR, the inhibitory effect of E priming was completely prevented. Pulsatile secretion of GnRH has been shown to be important in regulating pituitary LHβ mRNA expression (Dalkin et al., 1989). Accordingly, it is speculated that release of GnRH in ESR is severely damaged. However, in cyclic females, elevated LHβ mRNA levels were found in diestrus at which GnRH mRNA levels are high, GnRH secretion is low, and serum LH levels are also reduced. This condition is parallel to the findings of the present study.

In conclusion, the overall condition found in the ESR is similar to the endocrine characteristics found in diestrus of normal cyclic females. The increase in hypothalamic GnRH mRNA levels and the decrease in pituitary LHβ mRNA levels are common in both animals. The sterility of ESR may be due to the reduced release of GnRH as well as an alteration of GnRH gene expression. When administered to newborn female rats up to day 5, E causes the adult animals to be refractory to endogenous and exogenous steroids, which may be attributed to remodeling of the synapse responsible for developmental organization of the GnRH generating system. The specific action sites of E in the neonatal brain remain to be elucidated.

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References

Aihara M and Hayashi S (1989) Induction of persistent diestrus followed by persistent estrus is indicative of delayed maturation of tonic gonadotropin-releasing systems in rats. *Biol Reprod* 40: 96-101.

Barraclough CA (1961) Production of anovulatory, sterile rats by single injections of testosterone propionate. *Endocrinology* 68: 62-67.

Bloch GJ, Kurth SM, Akesson TR, and Micevych PE (1992) Estrogen-concentrating cells within cell groups of the medial preoptic area: Sex differences and co-localization within galanin-immunoreactive cells. *Brain Res* 595: 301-308.

Blum M (1989) Regulation of neuroendocrine peptide gene expression. In: Conn PM (ed), Neuroendocrine Peptide Methodology, Academic Press, New York, pp 3-18.

Boverdig J, Hermann H, and Bajusz S (1972) Ovulation induced by synthetic luteinizing hormone releasing factor in androgen-sterilized female rats. *J Endocrinol* 55: 207-208.

Brann DW and Mahesh VB (1991) Regulation of gonadotropin secretion by steroid hormones. *Front Neuroendocrinol* 12: 165-207.

Brann DW, O'Conner JL, Wade MF, Zamorano PL, and Mahesh VB (1993) Regulation of anterior pituitary gonadotropin subunit mRNA levels during the preovulatory gonadotropin surge: A physiological role of progesterone in regulating LH-β and FSH-β mRNA levels. *J Steroid Biochem Mol Biol* 46: 427-437.

Castro-Vazquez A and McCann SM (1975) Cyclic variations in the increased responsiveness of the pituitary to luteinizing hormone-releasing hormone (LHRH) induced by LHRH. *Endocrinology* 97: 13-19.

Chappel SC and Barraclough CA (1976) Plasma concentration changes in LH and FSH following electrochemical stimulation of the medial preoptic area or dorsal anterior hypothalamic area of estrogen- or androgen-sterilized rats. *Biol Reprod* 15: 661-669.

Cho BN, Seong JY, Cho H, and Kim K (1994) Progesterone stimulates GnRH gene expression in the hypothalamus of ovariectomized, estrogen-treated adult rats. *Brain Res* 652: 177-180.

Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156-159.

Dalkin AC, Haisenleder DJ, Ortolano GA, Ellis TR, and Marshall JS (1989) The frequency of gonadotropin-releasing hormone stimulation differentially regulates gonadotropin subunit messenger ribonucleic acid expression. *Endocrinology* 125: 917-924.

Davis EC, Shryne JE, and Gorski RA (1995) A revised critical period for the sexual differentiation of the sexually dimorphic nucleus of the preoptic area in the rat. *Neuroendocrinology* 65: 579-585.

Davis EC, Shryne JE, and Gorski RA (1996) Structural sexual dimorphisms in the anteroventral periventricular

nucleus of the rat hypothalamus are sensitive to gonadal steroids perinatally, but develop peripubertally. *Neuroendocrinology* 63: 142-148.

DePaolo LV and Barraclough CA (1979) Interactions of estradiol and progesterone on pituitary gonadotropin secretion: Possible sites and mechanism of action. *Biol Reprod* 20: 1173-1179.

Feinberg AP and Vogelstein B (1984) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 137: 266-267.

Gharib SD, Bowers SM, Need LR, and Chin WW (1986) Regulation of rat luteinizing hormone subunit messenger ribonucleic acids by gonadal steroid hormones. *J Clin Invest* 77: 582-589.

Gore AC and Roberts JL (1995) Regulation of gonadotropin-releasing hormone gene expression in the rat during the luteinizing hormone surge. *Endocrinology* 136: 889-896.

Gorski RA (1963) Modification of ovulatory mechanisms by postnatal administration of estrogen to the rat. *Am J Physiol* 205: 842-844.

Hayashi S and Aihara M (1989) Neonatal estrogenization of the female rat: A useful model for analysis of hypothalamic involvement of gonadotropin release. *Zool Sci* 6: 1059-1068.

Hayashi S, Aihara M, and Wakabayashi K (1991) Content and distribution pattern of luteinizing hormone-releasing hormone (LHRH) in the hypothalamus of neonatally estrogenized female rats. *Neurosci Res* 12: 366-378.

Herbison AE and Theodosis DT (1992) Localization of the estrogen receptors in preoptic neurons containing neurotensin but not tyrosine hydroxylase, cholecystokinin or luteinizing hormone- releasing hormone in the male and female rat. *Neuroscience* 50: 283-298.

Jacobson CD, Csernus VJ, Shryne JE, and Gorski RA (1981) The influence of gonadectomy, androgen exposure, or gonadal graft in the neonatal rat on the volume of the sexually dimorphic nucleus of the preoptic area. *J Neurosci* 1: 1142-1147.

Kalra SP and Kalra PS (1989) Do testosterone and estradiol-17β enforce inhibition or stimulation of luteinizing hormone releasing hormone secretion? *Biol Reprod* 41: 559-570.

Kim K, Lee BJ, Park Y, and Cho WK (1989) Progesterone increases messenger ribonucleic acid (mRNA) level in the hypothalamus of ovariectomized estradiol-primed prepubertal rats. *Mol Brain Res* 6: 151-158.

King JC, Tobet SA, Snavely FL, and Arimura AA (1980) The LHRH system in normal and neonatally androgenized female rats. *Peptides* 1: 85-100.

Matt DW, Sayles TE, Jih MH, Kauma SW, and Lu JKH (1993) Alterations in the postovariectomy increases in gonadotropin secretion in middle-aged persistent-estrous rats: Correlation with pituitary gonadotropin subunit gene expression. *Neuroendocrinology* 57: 351-358.

McEwen BS, Lieberburg I, Chaptal C, and Krey LC (1977) Aromatization: important for sexual differentiation of the neonatal rat brain. *Horm Behav* 9: 249-263.

Mennin SP, Kubo K, and Gorski RA (1974) Pituitary responsiveness to luteinizing hormone-releasing factor in normal and androgenized female rats. *Endocrinology* 95: 412-416.

Peterson SL, McCrone S, Keller M, and Shores S (1995) Effects of estrogen and progesterone on luteinizing hormone-releasing hormone messenger ribonucleic acid levels: Consideration of temporal and neuroanatomical variables. *Endocrinology* 136: 3604-3610.

Pinilla L, Trimio E, Garnelo P, Bellido C, Aguilar R, Gaytn F, and Aguilar E (1993) Changes in pituitary secretion during the early postnatal period and anovulatory syndrome induced by neonatal oestrogen or androgen in rats. *J Reprod Fertil* 97: 13-20.

Schwanzel-Fukuda M and Pfaff DW (1989) Origin of luteinizing hormone-releasing hormone neurons. *Nature* 338: 161-164.

Schwanzel-Fukuda M, Morrell MJ, and Pfaff D (1985) Ontogenesis of neuron producing luteinizing hormone-releasing hormone (LHRH) in the nervus terminalis of the rat. *J Comp Neurol* 238: 348-364.

Shupnik MA, Gharib SD, and Chin WW (1988) Estrogen suppresses rat gonadotropin gene transcription *in vivo*. *Endocrinology* 122: 1842-1846.

Terasawa E, Wiegand SJ, and Bridson WE (1980) A role for medial preoptic nucleus on afternoon of proestrus in female rats. *Am J Physiol* 238: E533-E539.

Wiegand SJ, Terasawa E, Bridson WE, and Goy RW (1980) Effects of discrete lesions of preoptic and suprachiasmatic structures in the female rat. *Neuroendocrinology* 31: 147-157.

Wray S and Hoffman GA (1986) Developmental study of the quantitative distribution of LHRH neurons within the central nervous system of postnatal male and female rats. *J Comp Neurol* 252: 522-531.

Zanisi M and Messi E (1991) Sex steroids and the control of LHRH secretion. *J Steroid Biochem Mol Biol* 40: 155-163.

Zmeili SM, Papavasiliou SS, Thorner MO, Evans WS, Marshall JC, and Landefeld TD (1986) Alpha and luteinizing hormone beta subunit messenger ribonucleic acids during the rat estrous cycle. *Endocrinology* 119: 1867-1869.

Zoeller RT and Young III W (1988) Changes in cellular levels of messenger ribonucleic acid encoding gonadotropin-releasing hormone in the anterior hypothalamus of female rats during the estrous cycle. *Endocrinology* 123: 1688-1689.

Zoeller RT, Seeburg PH, and Young III WS (1988) In situ hybridization histochemistry for messenger ribonucleic acid (mRNA) encoding gonadotropin-releasing hormone (GnRH): Effect of estrogen on cellular levels of GnRH mRNA in female rat brain. *Endocrinology* 122: 2570-2577.

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