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Effects of Immobilization Stress on Estrogen-Induced Surges of Luteinizing Hormone and Prolactin in Ovariectomized Rats

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Reproductive function has been known to be impaired by various kinds of physical and emotional stress, but the mechanism by which stress impairs the reproductive axis has not been clearly understood. In the present study, the effects of immobilization stress were studied on the surges of luteinizing hormone (LH) and prolactin (PRL) induced by 17β-estradiol (E_2) in ovariectomized rats. Two weeks after bilateral ovariectomy, animals were implanted with the capsule containing E_2 or vehicle at 1000 h (designated as d 0). Immobilization was started at 1000 h and continued to 2100 h on d 2. Blood samples were collected according to the time schedule by a jugular vein catheter procedure. Immobilization stress inhibited basal release of LH and abolished E_2-induced LH and PRL surges in ovariectomized (OVX) rats. Daily repeated immobilization (from 1200 h to 1800 h, 6 h/d) for 3 d also abolished LH and PRL surges when examined at 1800 h on d 2. Although daily repeated immobilization stress reduced E_2-induced PRL mRNA levels, this stress failed to change LHβ mRNA levels in the anterior pituitary as determined by Northern blot analysis. Gonadotropin-releasing hormone (GnRH) receptor mRNA levels in the anterior pituitary were lowered by immobilization stress in the OVX, E_2-treated group. Dopamine D2 receptor mRNA levels in the anterior pituitary of OVX, E_2-treated rats were significantly decreased at 1800 h, compared with those at 1000 h. However, immobilization prevented a decrease in dopamine D2 receptor mRNA levels at 1800 h. GnRH content was increased in the mediobasal hypothalamus by immobilization in the OVX, E_2-treated group, suggesting that GnRH release was inhibited. Interestingly, GnRH mRNA levels in the preoptic area-anterior hypothalamic area were suppressed by immobilization stress in OVX, E_2-treated rats when determined at 1800 h. Therefore, we concluded that immobilization stress blocks E_2-induced LH surge possibly by inhibiting synthesis and release of GnRH at the hypothalamic level, and an increase of dopaminergic activity via D2 receptor at the pituitary level might be involved in the stress blockage of E_2-induced PRL surge.

Key Words: Immobilization stress; luteinizing hormone; prolactin; gonadotropin-releasing hormone; gonadotropin-releasing hormone receptor; dopamine D2 receptor.

Introduction

It is well known that female reproduction can be impaired by various physical or emotional stressors *(1–3)*. Acute or chronic stresses alter gonadotropin secretion from the anterior pituitary *(4–7)*, possibly by altering gonadotropin-releasing hormone (GnRH) neuronal activity in the hypothalamus *(8,9)*. Recently, it has been reported that immobilization stress applied on the day of proestrus inhibited luteinizing hormone (LH) surge and ovulation subsequently in the cycling rat *(10)*. It is well known that the LH surge depends on the GnRH secretion from the hypothalamus. GnRH neuronal activity can be modulated by various inhibitory factors such as endogenous opioids *(11)*, cytokine *(12)*, γ-aminobutyric acid *(13)*, and corticotropin-releasing hormone (CRH) *(8)*, as well as stimulatory factors such as catecholamine *(14)*, Neuropeptide Y *(15)*, and excitatory amino acids *(16)*.

Prolactin (PRL) seems to play a permissive role in the regulation of gonadal function. Various types of stress stimulate basal secretion of PRL *(17)*, whereas PRL surge is abolished by stress in normally cycling *(18)* and ovariectomized (OVX), estrogen-treated rats *(19)*. Gala *(17)* presented a hypothesis suggesting an interaction between tuberoinfundibular dopamine secretion and a hypothalamic PRL-releasing factor in the generation of PRL surges and the differential effects of stress on PRL secretion. However, the mechanisms by which stress impairs surges for LH and PRL are not clearly understood.

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Previous studies mainly focused on the effect of stress on the release of LH and PRL under various steroid milieu. It is, however, assumed that a change in LH and PRL secretion might be owing to an alteration in the synthesis of LH and PRL as well as an alteration in the sensitivity to their hypothalamic modulators. At the hypothalamic level, alteration in GnRH neuronal activity by stress might lead to a change in LH release from the pituitary. In the present study, we attempted to demonstrate the effects of immobilization stress on E_2-induced LH and PRL surges in OVX rats and to elucidate the mechanism by which stress exerts alteration in LH and PRL surges. Therefore, the effects of immobilization stress on mRNA levels for LHβ subunit and PRL as well as mRNA levels for GnRH receptor and dopamine D2 receptor were evaluated in the anterior pituitary. At the hypothalamic level, the content of GnRH in the mediobasal hypothalamus (MBH) and GnRH mRNA levels in the preoptic area-anterior hypothalamic area (POA-AHA) were also determined.

Results

Time Course Profiles of Plasma LH, PRL, and Corticosterone Levels Induced by Immobilization Stress in OVX, Vehicle- or 17β-Estradiol-Treated Rats

The first experiment was performed to delineate the time course profiles of plasma LH, PRL, and corticosterone levels by immobilization stress in OVX, vehicle- (V) or 17β-estradiol (E_2)-treated rats. To confirm whether animals were stressed by immobilization, plasma corticosterone levels were determined. Corticosterone levels were immediately increased in response to stress and remained significantly ($p < 0.05$) high during immobilization stress, indicating an activation of the hypothalamo-pituitary-adrenal (H-P-A) axis (Figs. 1C and 2C). In OVX + V-treated rats, plasma LH levels were significantly decreased during immobilization stress although decreases between 1700 and 2000 h were not statistically significant (Fig. 1A). Acute response of PRL to stress was, however, a dramatic increase in release and this increase returned to the basal level within 2 h. After 1800 h, PRL levels tended to increase in immobilized animals (Fig. 1B).

The Silastic capsule containing E_2 was implanted into OVX rats at 1000 h on d 0. Because E_2 acts negatively on LH release except during the LH surge period, E_2 implantation lowered basal LH levels. E_2 induced LH surge around 1800 h on d 2, and this surge was extinguished thereafter. E_2 increased PRL release, inducing PRL surge around 1800 h on d 2. Both LH and PRL were immediately released in response to immobilization and returned to the basal level within 2 h (Fig. 2A, B). However, immobilization stress completely abolished both surges of LH and PRL.

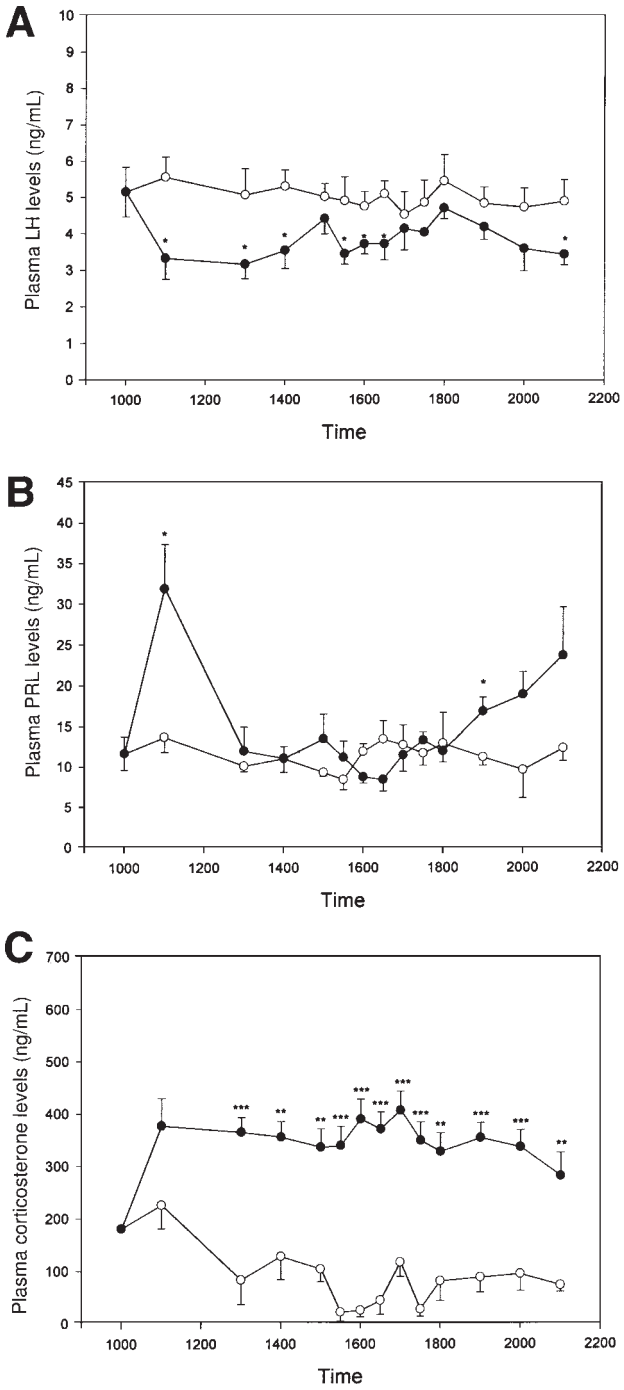

Fig. 1. Time course profiles of effect of immobilization stress on plasma LH **(A)**, PRL **(B)**, and corticosterone **(C)** levels in OVX and V-treated rats. Two weeks after ovariectomy, all animals were implanted with a Silastic capsule containing sesame oil at 1000 h. A heparinized catheter was implanted into the jugular vein of individual rats 1 d after implantation. Two days after implantation, the animals for the stressed group were immobilized for 11 h (1000 h–2100 h), and control animals were allowed to move freely for all the times. Blood samples (0.3 mL) were taken from the catheter according to the scheduled time and the same volume of heparinized saline was replaced at each sampling. Data points represent means ± SE. (s), control ($n = 6$); (d), immobolization ($n = 7$). *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ compared with control level.

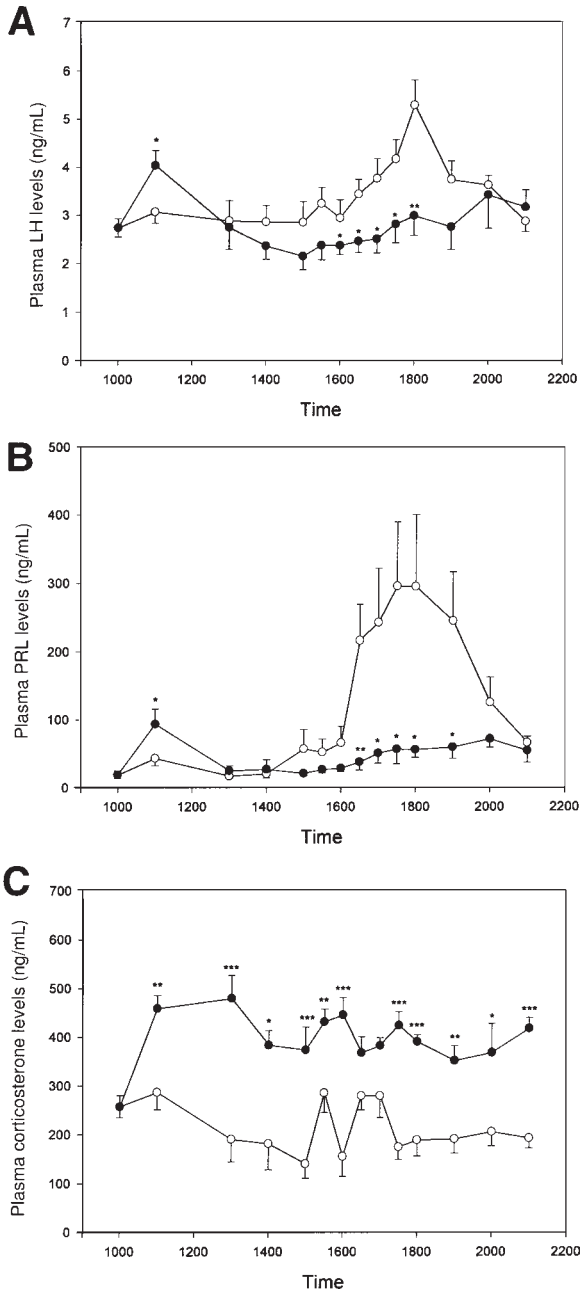

Fig. 2. Time course profiles of effect of immobilization stress on plasma LH **(A)**, PRL **(B)**, and corticosterone **(C)** levels in OVX and 17β-estradiol (E)-treated rats. The experimental scheme was the same as that described in Fig. 1 except E implantation instead of V. Data points represent means ± SE. (s), control ($n = 6$); (d), immobilization ($n = 8$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control level.

Effects of Daily Repeated Immobilization Stress for 3 d on E_2-Induced LH and PRL Surges in OVX Rats

To confirm the effect of immobilization on LH and PRL surges induced by E_2 in OVX rats, another regimen of immobilization stress was applied. Based on the first experiment, rats were sacrificed at 1800 h on d 2 when the peaks of LH and PRL surges were expected. The levels of LH and PRL at 1800 h were elevated by two- and sevenfold, respectively, compared with the levels of LH and PRL at 1000 h. However, daily repeated immobilization (6 h/d) for 3 d also clearly blocked both surges of LH and PRL. Interestingly, LH surge was reduced to the basal level by immobilization, but the PRL level remained higher than the basal level. Corticosterone levels were elevated by stress in both OVX + V- and OVX + E_2-treated rats (Fig. 3C).

Effects of Daily Repeated Immobilization for 3 d on Steady-State mRNA Levels of LHβ, PRL, and Receptors for GnRH and Dopamine in the Anterior Pituitary

This experiment was performed to determine whether synthesis of LH, PRL, and receptors for GnRH and dopamine at transcriptional level in the anterior pituitary was influenced by immobilization, thereby suppressing LH and PRL surges. Treatment with E_2 decreased LHβ mRNA levels whereas it increased PRL mRNA levels in OVX + V-treated rats (Fig. 4). However, immobilization stress did not alter LHβ mRNA levels in both OVX + V- and OVX + E_2-treated rats, whereas this stress significantly ($p < 0.05$) reduced PRL mRNA levels in OVX + E_2-treated rats.

Two bands (5.0 and 4.5 kb) for GnRH receptor mRNA are displayed in Fig. 4. GnRH receptor mRNA levels were significantly ($p < 0.05$) decreased by immobilization in OVX + E_2-treated rats. Changes in mRNA levels of D2 receptor, which are known to be involved in the regulation of PRL release, were inversely correlated to PRL release in OVX + E_2-treated rats (Fig. 4).

Effect of Daily Repeated Immobilization Stress for 3 d on GnRH Content in the MBH in OVX + E_2-Treated Rats

The basal GnRH content in the MBH at 1000 h was 90.61 ± 12.89 pg/mg in OVX + E_2-treated rats. The GnRH content in the MBH was 65.39 ± 12.51 pg/mg at 1800 h when the LH surge was induced by E_2 implantation. However, immobilized rats had about a twofold higher concentration of GnRH in the MBH compared with the control at 1800 h (Fig. 5).

Effect of Daily Repeated Immobilization Stress for 3 d on GnRH mRNA Levels in the POA-AHA in OVX + E_2-Treated Rats

Compared with GnRH mRNA levels at 1000 h, GnRH mRNA levels were significantly ($p < 0.05$) increased at 1800 h when the LH surge occurred, in the POA-AHA of OVX + E_2-treated rats. Daily immobilization stress for 3 d, however, suppressed the increase in GnRH mRNA levels at 1800 h in the POA-AHA (Fig. 6).

Discussion

The present study showed that immobilization stress reduced the basal release of LH and abolished E_2-induced

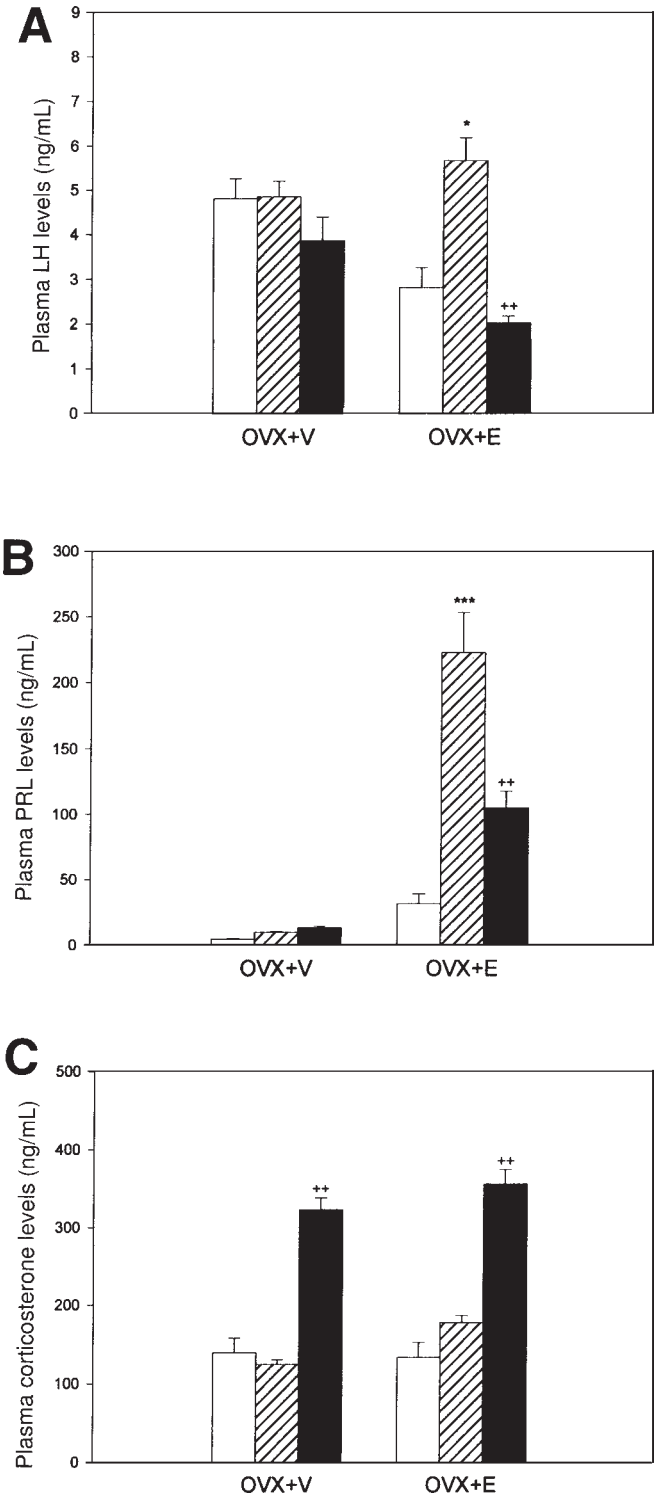

Fig. 3. Effect of daily repeated immobilization stress (6 h/d) for 3 d on E_2-induced LH **(A)** and PRL **(B)** surges and corticosterone **(C)** levels. For the control group, 2 wk after ovariectomy, animals were implanted with Silastic capsules containing V or E_2 at 1000 h. Two days later, animals were decapitated at 1000 or 1800 h. For the stressed group, animals were repeatedly immobilized for 3 d (1200–1800 h: 6 h/d). Each bar represents the mean ± SE (n = 5 to 6). (h), 1000 h; (▨) 1800 h (control); (j), 1800 h (immobilization). *$p < 0.05$, compared with 1000 h; + $p < 0.05$, compared with 1800 h.

LH surge in OVX rats. The early basal LH response to stress was inhibitory in OVX + V-treated rats, whereas it was stimulatory in OVX + E_2-treated rats. These results are in good agreement with reports that acute immobilization inhibited LH secretion in OVX + V-treated rats *(20)*, but, rather, increased LH secretion in OVX + E_2-treated rats *(6)*. Findings that exposure of cycling rats to inescapable foot shocks or restraint on the day of proestrus resulted in an inhibition of LH surge *(10,21)* support the present results. It seems that immobilization stress may inhibit LH surge at the level of release, since steady-state levels of LHβ subunit mRNA were not changed by immobilization in the present study. Because hypothalamic GnRH is a major regulator for LH release, immobilization stress might inhibit GnRH release from the hypothalamus, thereby inhibiting LH release. In this context, evaluation of GnRH content in the MBH gives rise to valuable information on GnRH release, because GnRH release into portal circulation cannot be easily determined. The present results showed that GnRH content in the MBH of OVX + E_2-treated rats tended to decrease during LH surge. GnRH content in the MBH was significantly higher at 1800h in immobilized rats compared with nonstressed rats, strongly suggesting an inhibition of GnRH release and subsequent LH release. A report that chronic intermittent foot shocks increased GnRH concentration in the median eminence of the anestrous ewe also supports the present results *(22)*.

Plasma corticosterone levels were elevated by immobilization stress throughout our study, implicating an activation of the H-P-A axis. It appears that the GnRH neuron can be affected by the paraventricular CRH neuron that is activated by immobilization stress. The facts that direct synaptic connections exist between CRH- and GnRH-containing neurons in the POA *(23)* and that CRH is inhibitory on GnRH neuron *(24)* suggest that a stress-induced decrease in GnRH release may be mediated by an activation of CRH neuron. However, Jeong et al. *(25)* reported that suppression of the reproductive axis occurs in CRH-deficient mice by immobilization stress, suggesting that CRH is not a required inhibitory regulator of GnRH release during the stress response. Therefore, they proposed that other central factors known to restrain LH secretion, such as endogenous opioids *(11,26,27)*, cytokine *(12)*, or arginine vasopressin *(28,29)*, may instead act together to inhibit LH secretion *(30)* in CRH-deficient mice. Thus, it is assumed that non-CRH pathways might also be involved in GnRH downregulation during stress. Blockage of E_2-induced LH surge in OVX rats by immobilization stress might be from a direct action of corticosteroids at the pituitary level. It has been demonstrated that cortisol and corticosterone could directly suppress basal as well as GnRH-stimulated LH release from rat anterior pituitary cells in vitro *(31,32)* and OVX rats in vivo *(33)*. The possibility that the inhibitory action of corticosterone might be exerted at higher brain

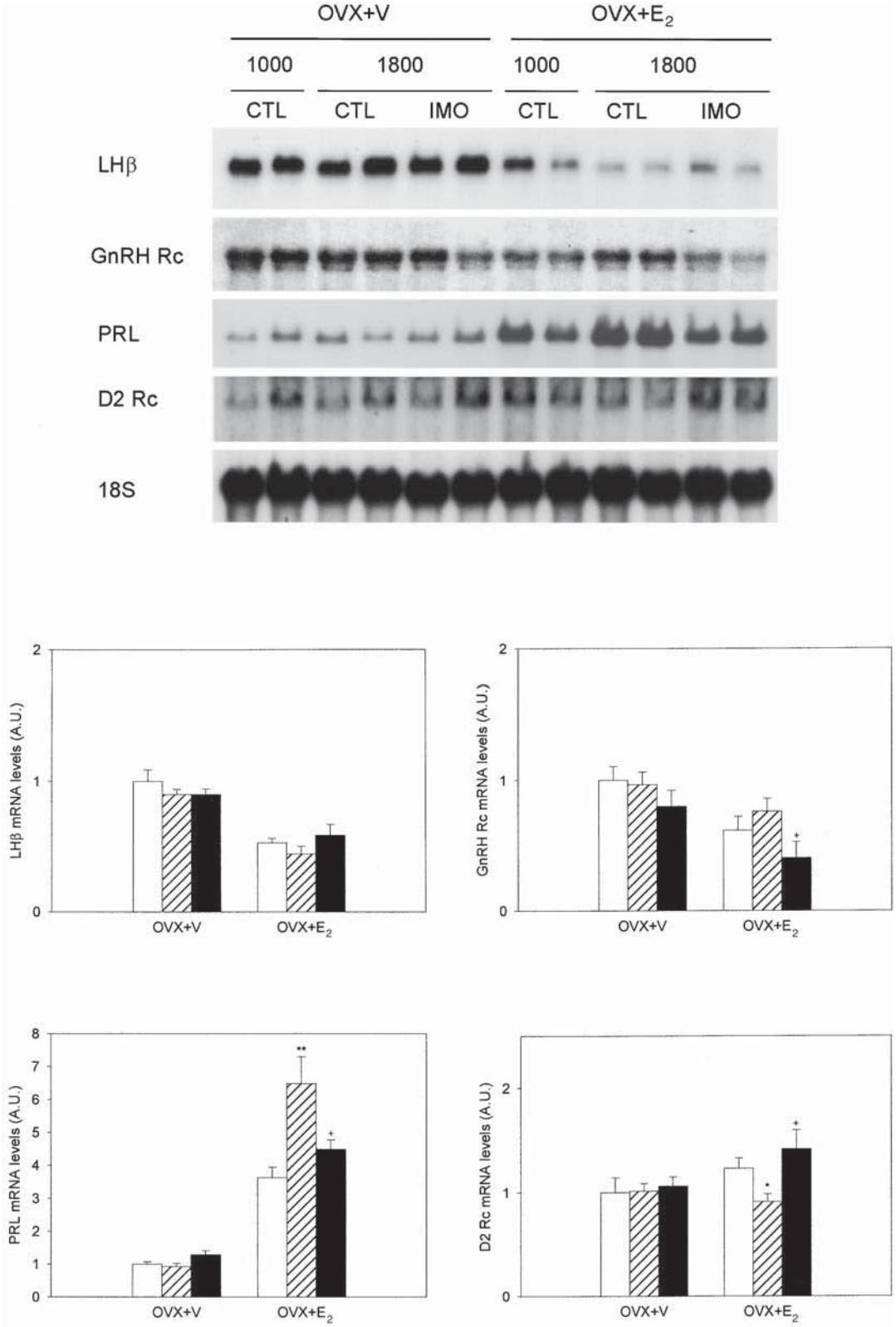

Fig. 4. Effect of daily repeated immobilization stress (6 h/d) for 3 d on mRNA levels of LHβ, GnRH receptor, PRL, and dopamine D2 receptor in the anterior pituitary. The experimental scheme is the same as that described in Fig. 3. **(Top)** Representative autoradiograms; **(bottom)** quantitative graphs. The intensity of bands was quantified by scanning densitometry. After normalization of each lane with 18S RNA content, results are presented as arbitrary units (A.U.). Each bar represents the mean ± SE ($n = 5$ to 6). 1000 h; (▨), 1800 h (control); (▩), 1800 h (immobilization). *$p < 0.05$, compared with 1000 h, + $p < 0.05$, compared with 1800 h. CTL, control; IMO, immobilization; GnRH Rc, GnRH receptor; D2 Rc, dopamine D2 receptor.

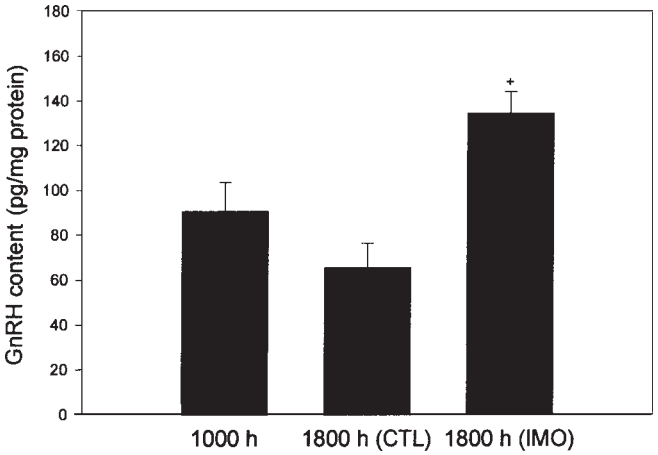

Fig. 5. Effect of daily repeated immobilization stress (6 h/d) for 3 d on GnRH content in the MBH. The GnRH content was determined by radioimmunoassay (RIA) and values were corrected with total protein content of each sample. Each bar represents the mean ± SE ($n = 4$ to 5). $^{+}p < 0.05$ compared with 1800 h (control).

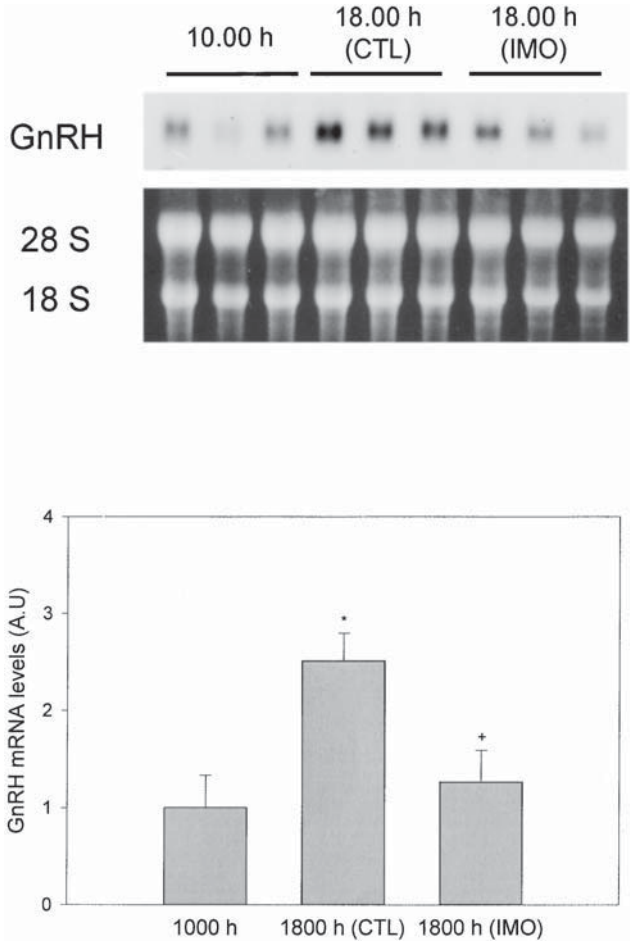

Fig. 6. Effect of daily repeated immobilization stress (6 h/d) for 3 d on GnRH mRNA levels in the POA-AHA. **(Top)** Representative autoradiogram; **(bottom)** quantitative graph. The ethidium bromide–stained gel before transfer, along with the integrity of 28S and 18S rRNA, is shown. The intensity of bands was quantified by scanning densitometry and the results are expressed as arbitrary units (A.U.). Each bar represents the mean ± SE (n = 4 to 5). $^{*}p < 0.05$, compared with 1000 h; $+ p < 0.05$, compared with 1800 (control).

centers, including the hypothalamus, cannot be ruled out. Smith et al. *(34)* have demonstrated that cortisol acetate implanted in the MBH of rats for an extended period prevented normal onset of puberty, most likely by an inhibition of GnRH release.

The present study shows that GnRH mRNA levels in the POA-AHA increased at 1800 h when the LH surge occurred, in good accordance with the findings of Zoeller and Young *(35)* and Park et al. *(36)*. However, daily repeated immobilization for 3 d significantly reduced GnRH mRNA levels at the same hour. The mechanism by which immobilization reduces GnRH gene expression cannot be clearly explained. However, it can be assumed that CRH activated by immobilization stress might interfere with the GnRH neuron, thereby inhibiting GnRH mRNA expression, although evidence to support this notion is not available. There is a possibility that stress-induced corticosterone in our study might repress GnRH gene expression, because glucocorticoids inhibited GnRH gene expression directly via glucocorticoid receptor in GT1 hypothalamic cell lines *(37–40)*.

Interestingly, in response to stress, mRNA levels of GnRH receptor in the anterior pituitary were significantly decreased during the period of E_2-induced LH surge. The number of GnRH receptors on the gonadotropes is an important determinant for pituitary responsiveness to GnRH. It is known that GnRH receptors on the gonadotropes are homologously regulated by GnRH itself *(41,42)*. The administration of pulsatile GnRH upregulates GnRH receptor number *(43)* and its gene expression *(44)* in the anterior pituitary. Recently Lin and Conn *(45)* and Cheon et al. *(46)* reported that even continuous administration of GnRH upregulates GnRH receptor mRNA levels in primary of anterior pituitary cultured cells within several hours. It has been reported that glucocorticoids modulate gonadotropin secretion by altering pituitary responsiveness to GnRH in an E_2-dependent manner *(32)*. However, evidence that glucocorticoids can modulate GnRH receptor gene expression has not been available, although the glucocorticoid/progesterone response element was found in GnRH receptor gene *(47)*. Therefore, a reduction in GnRH receptor mRNA levels in the anterior pituitary by immobilization seems to be the result of an inhibition of GnRH secretion from the hypothalamus.

In the present study, plasma PRL levels were acutely increased within an hour in response to immobilization stress and returned to basal levels thereafter in OVX + V-treated rats. On the other hand, immobilization stress clearly blocked E_2-induced PRL surge, although early PRL release was also observed in response to stress in OVX + E_2-treated

rats. The rapid termination of early PRL response to stress seems to be owing to the suppressive effect of stress-induced corticosterone *(48)*. However, it is not conceivable that elevated corticosterone is responsible for stress-induced blockage of PRL surge, because adrenalectomy did not prevent stress-induced blockage of PRL surge in rats *(49,50)*. Immobilization stress may block PRL surge, in part, by inhibiting PRL synthesis at the level of transcription because immobilization significantly depressed PRL mRNA levels during the period of PRL surge in the present study. It has been shown that dopaminergic binding sites *(51)* and dopamine levels in portal blood *(52)* are depressed on the day of proestrus in rats, and that the concentrations of dopamine and its major metabolite, 3,4-dihydroxyphenylacetic acid, are decreased prior to proestrous surge *(53)*, allowing increased secretion of PRL into the peripheral circulation. The treatment of pimozide, a dopamine antagonist, prevented stress-induced blockage of PRL surge in rats *(54)*. In the present study, D2 receptor mRNA levels in the anterior pituitary were significantly decreased when PRL surge occurred, compared with those at 1000 h. However, immobilization stress reversed D2 receptor mRNA levels. Morehead et al. *(19)* reported that the inhibitory effect of restraint stress on PRL surge was accompanied by an increase in tuberoinfundibular dopamine neuronal activity. Therefore, it seems that the dopaminergic system might be responsible for stress-induced blockage of PRL surge although dopamine levels were not determined in our study.

In conclusion, the present data show that immobilization stress might block E_2-induced LH surge in OVX rats by inhibiting synthesis of GnRH at the level of transcription and by inhibiting the release of GnRH from the hypothalamus. Increased dopaminergic action via D2 receptor in the pituitary might be involved in the stress blockage of E_2-induced PRL surge.

Materials and Methods

Animals and Experimental Design

The following procedures were reviewed by the Committee for the Care and Use of Laboratory Animals in Yonsei University according to the Guidelines and Regulations for Use and Care of Animals in Yonsei University. Adult female Sprague-Dawley rats (250–300 g) were supplied from Division of Laboratory Animal Medicine, Yonsei University College of Medicine. Animals were housed in temperature (22°C)- and humidity (55%)-controlled conditions under a 12-h light and 12-h dark photocycle (light on at 0600 h) with food and water supplied ad libitum. All animals were OVX under light ether anesthesia, and 2 wk later the animals were used.

Experiment 1

Time course profiles of LH, PRL, and corticosterone levels in systemic circulation induced by immobilization stress were examined. Silastic capsules (30 mm in length, 1.575-mm id, 3.175-mm od; Dow Corning, Silastic Medical Grade Tubing) containing 17β-estradiol (180 μg/mL in sesame oil) or vehicle was implanted subcutaneously near the neck in OVX rats at 1000 h on the day of the experiment, designated as d 0. One day after implantation (d 1), a catheter was implanted into the right jugular vein for blood sample collection on the next d. On d 2, immobilization was applied by placing the rat in an acryl restrainer (purchased from Myungjin, Korea) from 1000 to 2100 h for 11 h. For the control group, animals were allowed to move freely. Blood samples (0.3 mL) were taken from the catheter according to the scheduled time, and the same volume of heparinized saline was replaced at each sampling.

Experiment 2

The experiment was designed to examine whether immobilization stress affects plasma LH and PRL surges induced by E_2. In addition, LHβ and PRL mRNA levels and GnRH receptor and dopamine D2 receptor mRNA levels in the anterior pituitary and GnRH content and mRNA levels in the hypothalamus were examined. E and V were treated to OVX rats according to the same method described in the first experiment. Two hours (1200 h, d 0) after steroid implantation, immobilization was performed on the experimental group until 1800 h. The next day (d 1), the same stress was applied to the same animals for 6 h (1200–1800 h). On d 2, the experimental group was immobilized a third time. No immobilization stress was applied to the control animals. Some of the control animals were sacrificed by decapitation at 1000 h, when the surges of two hormones do not begin yet. At 1800 h, when the surge of both LH and PRL occurred, based on the first experiment, all animals were sacrificed by decapitation. The hypothalamic fragments including the POA-AHA and MBH were readily isolated. Because GnRH-synthesizing cell bodies signals are concentrated in the POA-AHA *(55)*, POA-AHAs were used to determine GnRH mRNA levels. GnRH tissue content was determined in the MBH. The boundaries of the POA-AHA were as follows: caudally, the posterior border of the optic chiasm; frontally, the apex of the isosceles triangle–shaped region; laterally, the lateral hypothalamic sulci; and transversely, at a depth of 2 to 3 mm *(56)*. The dissection of the MBH extended to the anterior border of the mamillary body as previously described *(57)*. MBH fragments were homogenized with 0.02 *N* acetic acid and centrifuged at 10,000*g* for 20 min, and the supernatants were kept at –20°C for GnRH RIA. Trunk bloods were collected, and anterior pituitaries were removed and frozen at –70°C until use.

Determination of Plasma LH, PRL, and Corticosterone Levels and GnRH Content

Blood samples were immediately centrifuged at 3000*g* for 10 min, and the plasma was frozen at –20°C until use. LH and PRL levels were assayed using double antibody RIA reagents kindly provided by the National Hormone and

Pituitary Program, NIDDK. NIDDK-rLH-I-9 and NIDDK-rPRL-I-6 were iodinated by the chloramine-T method. The antisera were prepared with NIDDK-anti-rLH-S-10 and NIDDK-anti-rPRL-S-9, and the reference preparations were NIDDK-rLH-RP-2 and NIDDK-rPRL-RP-3 for LH and PRL, respectively. The intra- and interassay coefficients of variation of RIA were 5–7 and 8–10%, respectively. Corticosterone levels were determined by fluorometric assay *(58)*.

GnRH content in the MBH fragments was measured by GnRH RIA using the Chen-Ramirez GnRH antiserum CRR13B73 (generously provided by Dr. V.D. Ramirez, University of Illinois) at a final dilution of 1:180,000 *(56)*. Protein concentrations were determined by protein assay kit (Bio-Rad, Hercules, CA).

Total RNA Extraction

Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method *(59)*. Briefly, each pituitary was homogenized in 600 μL of denaturing solution containing 4 *M* guanidinium thiocyanate, 25 m*M* sodium citrate (pH 7.0), 5% *N*-lauryl sarcosine, and 0.1 *M* 2-mercaptoethanol. Sixty microliters of 2 *M* sodium acetate (pH 4.0), 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°C for 20 min and precipitated with ethanol at –20°C. After washing with 75% ethanol, the RNA pellet was dried under vacuum and dissolved in 20 μL of sterilized distilled water. RNA content was then quantified at A_{260} absorbance. The optical density ratio of A_{260}:A_{280} ranged from 1.8 to 2.0.

Northern Blot Hybridization Assay

Pituitary and hypothalamic total RNAs (20 μg) were dissolved in distilled water and denatured in 50% formamide, 6.2% formaldehyde, 20 m*M* 3-(*N*-morpholino)propanesulfonic acid, 5 m*M* sodium acetate, and 1 m*M* EDTA at 60°C for 5 min. Electrophoresis was performed at 100 V for 1.5 h in a submarine 1.2% agarose gel. After RNAs were transferred to a Nytran filter (pore size: 0.45 μm) for 18 h by diffusion blotting, the filter was dried and baked at 80°C for 2 h.

Prehybridization was carried out at 42°C for 2 h in a heat-sealable plastic bag (Kapak) with hybridization buffer consisting of 50% deionized formamide, 5X SSPE, 5X Denhardt's solution (1X Denhardt's solution: 0.02% polyvinylpyrrolidone, 0.02% Ficoll, and 0.02% bovine serum albumin), 0.1% sodium dodecyl sulfate (SDS), and 2 mg of heat-denatured salmon sperm DNA. After addition of ^{32}P-labeled LHβ cDNA probe made using a Rediprime random primer labeling kit (Amersham Life Science, UK), hybridization was performed at 42°C for 20 h. The Nytran membranes were washed three times with 2X saline sodium citrate (SSC) and 0.1% SDS at room temperature for 5 min, followed by a second washing with 0.1X SSC, 0.1% SDS, and 5 m*M* EDTA (pH 8.0) at 42°C for 15 min. The membranes were then dried and exposed to X-ray film (Fuji) at –70°C for 1 or 2 d.

To remove probe from the blot membrane for reuse, a solution of 0.1X SSPE/0.5% SDS was heated to boiling, and the membrane was incubated in the solution for 15 min. Complementary DNA probe labeled with ^{32}P from PRL cDNA in plasmid SP65 was rehybridized on the same membrane and washed as previously described. GnRH receptor cDNA was obtained from the rat pituitary RNA *(60)*. The pGEM4Z plasmid containing rat GnRH receptor cDNA was linearized by *Hind*III. Dopamine D2 receptor cDNA *(61)* inserted in pGEM3Z was a kind gift from Dr. Wan Sung Choi (Gyeongsang National University). Antisense cRNA probes for GnRH receptor and D2 receptor were synthesized in the presence of ^{32}P-UTP using SP6 and T7 RNA polymerase, respectively. Hybridization buffer was the same as already described . Hybridization was performed at 62°C for 20 h, and then the membranes were washed twice with 2X SSC and 0.1% SDS at room temperature for 20 min, followed by 0.07X SSC, 0.5% SDS, and 5 m*M* EDTA (pH 8.0) at 62°C for 10 min. The same membrane was then rehybridized with 18S cDNA probe under the same conditions except the hybridization temperature used was 42°C. The rehybridized membrane was washed twice with 2X SSC and 0.1% SDS at room temperature for 20 min, followed by 0.1X SSC and 0.1% SDS at 42°C for 20 min. Finally, the membrane was rehybridized at 42°C for 14 h with 18S cDNA as an internal control.

Template DNA for GnRH was the rat cDNA clone (generously provided by Dr. Kelly E. Mayo, Northwestern University, USA) inserted into pGEM4 vector (Promega, USA). RNA probe was labeled with ^{32}P-UTP using SP6 RNA polymerase. Hybridization and washing procedures were similar to those for GnRH receptor and D2 receptor.

Statistical Analysis

The data were analyzed by unpaired Student's *t*-test. A probability level of $p < 0.05$ was selected for the determination of statistically significant differences.

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