Hypothalamic Gonadotropin-Releasing Hormone Gene Expression during Rat Estrous Cycle

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Abstract. Expression of gonadotropin-releasing hormone (GnRH) gene in the hypothalamus of female rats was studied by the quantitative reverse transcription-polymerase chain reaction (RT-PCR) method. During the estrous cycle, the GnRH mRNA level did not change from diestrus I to II, and then increased with a peak at 1100 h on proestrus. In the afternoon of proestrus, GnRH mRNA decreased rapidly with a nadir at 1600 h, and thereafter increased again and reached a peak at 1100 h on estrus. Since hypothalamic GnRH mRNA was found to be increased by subcutaneous implantation of estradiol-containing silastic tubing in ovariectomized rats, the peak of GnRH gene expression in the proestrus morning might be due to an increase in circulating estrogen in this phase of the estrous cycle. The surge of luteinizing hormone in the proestrus afternoon and the subsequent increase in GnRH mRNA were completely blocked by the injection of MK801, an antagonist for NMDA receptors, suggesting that the excitation of GnRH neurons leads to an increase in GnRH gene expression. This was further supported by the in vitro observation that high K+-induced membrane depolarization markedly increased GnRH mRNA in hypothalamic slices. This increase in GnRH mRNA due to neuronal excitation does not seem to require newly synthesized proteins because anisomycin, a protein synthesis inhibitor at the level of translation, did not affect GnRH gene expression. These results suggest that the hypothalamic GnRH mRNA level reached two peaks during the rat estrous cycle, i.e., in proestrus morning and estrus morning, and that estrogen and GnRH neuronal excitation play pivotal roles in regulating GnRH gene expression.

Key words: GnRH gene expression, Rat estrous cycle, Estrogen, Neuronal excitation, RT-PCR

GONADOTROPIN-releasing hormone (GnRH) is one of the most important molecules in the reproductive system [1]. Virtually all the mammalian species have GnRH molecules of the same structure. Cloned cDNAs originating in the rat hypothalamus and placenta consist of 4 exons, and the second exon encodes for GnRH [2]. The coding sequence translates for a 92 amino acid precursor protein, which delivers GnRH and also a 56 amino acid peptide termed GnRH-associated peptide (GAP) by processing [3]. Since hypothalamic GnRH neurons are regarded as a final common pathway in the brain control of reproductive function, GnRH gene expression should be a useful indicator to show how the brain integrates and appraises internal and external information in performing reproduction. In this context, several investigators attempted to demonstrate changes in GnRH gene expression during the rat estrous cycle [4–7], but the results were controversial. One of the difficulties in detecting GnRH mRNA is that the total number of GnRH neurons is estimated to be about 1,300 in the rat hypothalamus [8] and the amount of GnRH mRNA expressed is extremely small. Most of the studies...
have therefore been done by means of in situ hybridization, which is not appropriate for quantitative analysis. Although no consistent profile of GnRH gene expression during the rat estrous cycle has yet been established, several endocrinological and neurological factors may be involved in regulating GnRH gene expression. Among these factors, estrogen and membrane depolarization attract most attention. Neither estrogen receptors in GnRH neurons nor the estrogen response elements in 5'-flanking region of GnRH gene have been identified. Nevertheless, estrogen is supposed to affect GnRH gene expression through neurons containing estrogen receptors, since GnRH release largely depends on the blood estrogen level [9-12]. On the other hand, it is suggested that both the GnRH gene and early genes such as c-fos and c-jun are coexpressed in GnRH neurons during the luteinizing hormone (LH) surge due to the excitation, or membrane depolarization of, GnRH neurons in the proestrous evening [13, 14]. However, a signal transduction cascade from membrane depolarization to GnRH gene expression remains to be clarified.

In the present study, to elucidate the mechanisms regulating hypothalamic GnRH gene expression in female rats, the quantitative reverse transcription-polymerase chain reaction (RT-PCR) method, which makes it possible to amplify and analyze minute amounts of mRNA, was developed. By means of this analytical method, first the profile of GnRH mRNA expression in the hypothalamus during the rat estrous cycle was examined. The effect of estrogen on GnRH gene expression was also evaluated in ovariectomized rats. Further, to investigate how neuronal excitation is involved in the activation of GnRH gene expression, an in vitro perfusion system for hypothalamic slices was established and the effect of high K+-induced membrane depolarization on GnRH gene expression was studied.

**Materials and Methods**

**Animals**

Adult female Wistar-Imamichi rats were housed in a temperature (23 ± 1 °C) and light-controlled room (lights on 0500–1900 h) and received laboratory chow and water ad libitum. Stages of the estrous cycle were monitored by daily sampling of vaginal smears, and only those demonstrating at least two consecutive 4-day cycles were used in this study.

**Sampling of hypothalamic tissue during an estrous cycle**

Rats were sacrificed by decapitation at 1100 and 1800 h on estrus, diestrus I and II, and at 1100, 1400, 1600, 1800 h on proestrus (n=3 for each time point). The brain was immediately removed and the entire hypothalamus was cut, bordered anteriorly by the optic chiasma, laterally by the hypothalamic fissures and posteriorly by the mamillary body, was dissected out. Its depth from the basal surface of the hypothalamus was 3 mm. After the dissection, the hypothalamic block was frozen in liquid nitrogen and stored at −80 °C until RNA analysis.

**E2 implantation**

Rats were ovariectomized under ether anesthesia. Two weeks later, they were implanted subcutaneously with estradiol-containing silastic tubing (10 mm length, 3 mm outer diameter; 70% cholesterol/30% 17β-estradiol, Sigma, MO., USA) at 1200 h, and this day was designated as Day 0. By means of this treatment, the serum estradiol level rose to 100 pg/ml, which was nearly equal to the level at around noon on proestrus [15]. At 1100 and 1600 h on Days 1 and 5, rats were sacrificed and the hypothalamus was removed and stored (n=3 in each group).

**Blockade of LH surge by MK801**

To block the LH surge on proestrus, noncompetitive NMDA receptor antagonist MK801 ((+)/-5-methyl-10, 11-dihydro-5H-dibenz(o, d) cyclo-heptin-5, 10-imine malate; Research Biochemicals, MA., USA) was injected subcutaneously (0.2 mg/kg BW) at 1200 and 1500 h into proestrus rats without anesthesia (n=4). The control rats (n=4) received saline. At 1600 and 2000 h, the rats were decapitated and the brains dissected out. Trunk blood was collected at the same time and the serum was separated and stored at −20 °C for LH determination. The serum concentration of LH was measured by the double antibody
radioimmunoassay method with materials supplied by the NIDDK. The reference standard for LH assay was NIDDK-rLH-RP-3.

Perfusion of hypothalamic slices

Ovariectomized rats were killed by decapitation at 1100 h, and the brain was quickly removed and trimmed. The hypothalamic slices (400 μm thick) including the preoptic area were dissected out, and four slices prepared from each brain were placed in a perfusion chamber (800 μl volume) and incubated at 37 °C. The slices were perfused at a flow rate of 500 μl/min with artificial cerebrospinal fluid (standard medium, 124 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1.3 mM MgSO₄, 1.25 mM KH₂PO₄, 26 mM NaHCO₃, 10 mM glucose, and pH 7.4), supplemented with amino acids at cerebrospinal fluid levels [16], and gassed continuously with 95% O₂/5% CO₂. After preincubation for 1 h, the slices were perfused with high K⁺-medium (56 mM KCl, and the other components were the same as those in the standard medium except for 73 mM NaCl) or the standard medium for 2 h. Perfusion with the high K⁺-medium was performed either in the presence or absence of anisomycin (10 mM; Sigma, MO., USA), a protein synthesis inhibitor at the translation level. After these treatments, slices were further perfused with the standard medium for an additional 1 h. At the end of the perfusion experiment, the slices were collected and stored at -80 °C until RNA analysis.

Extraction of total RNA and RT-PCR

Total RNA was extracted by the method with guanidium thiocyanate followed by centrifugation in cesium chloride solutions [17]. For RT-PCR, we used a Gene Amp™ RNA PCR kit (PERKIN ELMER, NJ., USA). For the template to synthesize single stranded DNA, 1 μg of total RNA was incubated at 42 °C for 60 min in a final volume of 20 μl with 50 U cloned Moloney murine leukemia virus reverse transcriptase. After the reaction, 5 μl of the reaction mixture was electrophoresed on a 1% agarose gel in 1×TAE buffer [17] and the bands were detected by ethidium bromide (2 mg/ml) staining. After washing with distilled water, the agarose gel was photographed. The pictures were scanned with an image scanner (GT-6500, EPSON, Tokyo, Japan) and the relative intensity of the ethidium bromide fluorescence of each band was analyzed with NIH Image software. The data were statistically evaluated by one-way analysis of variance followed by Duncan's multiple range test. The level of significance was set at P<0.05.

Results

Relative amount of PCR products from graded doses of hypothalamic total RNA

The linear relationship between the graded doses of template (2⁻⁵⁻⁻²² μg of hypothalamic total RNA) and the relative amount of RT-PCR products is shown in Fig. 1. This linearity was maintained between 22 and 36 cycles. These results indicated that GnRH mRNA could be quantitatively determined within the ranges shown here by the RT-PCR method employed in the present study.
Changes in GnRH mRNA expression during the rat estrous cycle

GnRH gene expression in the hypothalamus was determined throughout the 4-day estrous cycle by RT-PCR. Since the expression of the G3PDH gene did not show significant changes throughout the estrous cycle (data not shown), the data were expressed as the relative amounts of PCR products. As shown in Fig. 2, GnRH gene expression did not change during diestrus I and II, and then increased with a peak at 1100 h on proestrus. In the afternoon of proestrus, GnRH gene expression decreased rapidly with a nadir at 1600 h. Thereafter, GnRH gene expression increased again and reached a peak at 1100 h on estrus.

Effect of estrogen on GnRH gene expression

Rats were left ovariectomized for 2 weeks, then supplemented with estradiol for 1 or 5 days. As shown in Fig. 3, estradiol increased hypothalamic GnRH gene expression in the ovariectomized rat gradually with time after treatment.

Effect of MK801 on serum LH and GnRH gene expression

MK801 completely blocked the LH surge on proestrus (Fig. 4). In the saline-treated rats, GnRH gene expression at 2000 h was significantly higher than that at 1600 h (Fig. 5), in agreement with the result shown in Fig. 2. In the MK801-treated rats, although the GnRH mRNA level at 1600 h was not different from that in the saline-treated rats, the increase in gene expression at 2000 h observed in the control rats was completely abolished.

Effect of membrane depolarization on GnRH gene expression in vitro

As shown in Fig. 6, perfusion of hypothalamic slices with high K⁺-medium for 2 h caused a significant increase in GnRH mRNA. Anisomycin did not affect the stimulatory effect of high K⁺-medium on GnRH gene expression.
Fig. 3. Effect of estradiol treatment for 1 and 5 days on hypothalamic GnRH gene expression in ovariectomized rats. The ovariectomized control (OVX) value was defined as 100%. Each column and vertical bar represent the mean ± SEM (n=6). The asterisk indicates a significant difference at P<0.05 compared to OVX.

Fig. 4. Effect of MK801 on serum LH concentration in proestrous rats. Each column and vertical bar represent the mean ± SEM (n=4). The asterisk indicates a significant difference at P<0.01 compared to the value at 1600 h of the corresponding group.

Fig. 5. Effect of MK801 on hypothalamic GnRH gene expression in proestrous rats. The value for saline-treated animals at 1600 h was defined as 100%. Each column and vertical bar represent the mean ± SEM (n=4). The asterisk indicates a significant difference at P<0.01 compared to the value for 1600 h of the corresponding group.

Fig. 6. Effect of high K⁺-medium with or without anisomycin on GnRH gene expression in the hypothalamic slices in vitro. The control value was defined as 100%. Each column and vertical bar represent the mean ± SEM (n=4). The asterisks indicate significant differences at P<0.05 compared to the control.
Discussion

The present study disclosed two significant peaks of GnRH gene expression during the rat estrous cycle, i.e., at 1100 h on proestrus and 1100 h on estrus. Because the profile of GnRH gene expression from diestrus to proestrous morning can be superimposed on that of serum estrogen levels [5], it is possible that the GnRH gene expression during this period depends on estrogen. This is supported by the present observation that estrogen administration to ovariectomized rats for 5 days stimulated GnRH gene expression, although a significant effect was detected for only 1 day. This discrepancy between intact and ovariectomized rats in the time course of the estrogen effect on gene expression might be due to the difference in the estrogen sensitivity of the brain. A similar facilitatory effect of estrogen on GnRH gene expression has also been reported by others [9-12]. The increase in GnRH gene expression during this period of estrous cycle may therefore be probably ascribed to a gradual increase in peripheral estrogen secreted from the preovulatory follicles.

The increase in GnRH gene expression in the proestrous evening occurred following the LH surge. The LH surge is supposed to be associated with maximal excitation of GnRH neurons, since the expression of c-Fos and c-Jun in GnRH neurons is enhanced during this period [13, 14]. It has also been shown that the GnRH gene possesses the AP-1 site for the Fos-Jun complex upstream [2, 13, 14]. Taken together, the excitation of GnRH neurons may lead to an increase in GnRH gene expression. To confirm this, GnRH gene expression was examined after the injection of MK801, an NMDA receptor antagonist, that is known to block the LH surge [27]. An injection of MK801 on proestrus completely blocked the LH surge with a resultant suppression of GnRH gene expression.

To further analyze a linkage between membrane depolarization and transcriptional activation, an in vitro perfusion system using brain slices was employed. Perfusion of hypothalamic slices with high K+-medium resulted in an increase in GnRH gene expression, suggesting that hypothalamic slices with high K+-medium resulted in an increase in GnRH gene expression, suggesting that membrane depolarization itself, without mediation through NMDA receptors, could activate transcription of the GnRH gene. Moreover, anisomycin failed to impair the high K+-induced increase in GnRH mRNA, which indicates that newly synthesized proteins are not a prerequisite for the expression of the GnRH gene. It was recently reported that stress-induced corticotropin-releasing hormone (CRH) gene expression in the rat hypothalamus is not impaired by antisense oligonucleotides to c-fos and c-jun, which are co-expressed in CRH neurons [28]. These results suggest that immediate early genes, which are commonly expressed during neuronal excitation, are not always necessary for the transcriptional activation of the other genes. On the other hand, it is well known that membrane depolarization triggers a cascade of intracellular events including the activation of protein kinase C, Ca2+/calmodulin dependent protein kinase, and a cAMP dependent pathway [29]. Through these signal transduction pathways, the membrane depolarization would stimulate GnRH gene expression.

Although the reported data regarding a profile of GnRH peptide in the hypothalamus during the rat estrous cycle are still not fully consistent, general agreement is that GnRH increases from diestrus II to the proestrus morning, then declines due to a massive release to induce the LH surge, and remains at a low level until the next diestrus [30]. The increase in GnRH mRNA from diestrus to proestrus morning therefore seems to contribute to the increase in GnRH peptide synthesis required for the subsequent release of the peptide.
Interestingly, estrogen stabilizes mRNAs coding several peptides such as ovalbumin, conalbumin and vitellogenin, and this effect of estrogen is not mediated by estrogen receptors [31]. The serum estrogen level in the proestrous morning is much higher than the other periods of the estrous cycle, and thus GnRH mRNA during this phase may be stabilized and contribute to producing a large amount of the peptide.

In summary, a quantitative RT-PCR method was employed to determine changes in GnRH gene expression in the rat hypothalamus during an estrous cycle. It was demonstrated that GnRH gene expression comprises 2 peaks, one in the proestrous morning and one in the estrous morning. Estrogen and the membrane excitation of GnRH neurons may contribute to increasing GnRH gene expression. The latter is evoked at least partially through excitatory amino acid receptors but not mediated by immediate early genes. The relationship between transcription and the translation of the GnRH gene remains to be elucidated.

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References


