Reproductive Sciences
2017, Vol. 24(2) 227-233

DOI: 10.1177/1933719116653679
journals.sagepub.com/home/rsx
SAGE

Expression of GnRH and Kisspeptin in Primary Cultures of Fetal Rat Brain

Unurjargal Sukhbaatar, MD, PhD[1], Haruhiko Kanasaki, MD, PhD[1], Tselmeg Mijiddorj, MD, PhD[1], Aki Oride, MD, PhD[1], Tomomi Hara, MD[1], Takaya Yamada, PhD[2], and Satoru Kyo, MD, PhD[1]

Abstract

Genetic studies in humans or in vivo studies using animals have shown that kisspeptin released from the hypothalamus controls secretion of gonadotropin-releasing hormone (GnRH) from GnRH neurons, and subsequently GnRH induces gonadotropin secretion from the anterior pituitary. Kisspeptin did not stimulate GnRH expression in the GnRH-producing cell line GT1-7. Thus, we cultured GnRH and kisspeptin neurons from whole fetal rat brain and examined the regulation of GnRH and kisspeptin. Expression of GnRH messenger RNA (mRNA) was unchanged by estradiol (E2) treatment in these primary cultures. In contrast, kisspeptin mRNA expression was increased 2.00 $\pm$ 0.23-fold by E2 treatment. When these cultures were stimulated by kisspeptin-10, GnRH mRNA was significantly increased up to 1.51 $\pm$ 0.35-fold. Expression of GnRH mRNA was also stimulated 1.84 $\pm$ 0.33-fold by GnRH itself. Interestingly, kisspeptin mRNA was significantly increased up to 2.43 $\pm$ 0.40-fold by kisspeptin alone. In addition, kisspeptin mRNA expression was significantly increased by stimulation with GnRH (1.46 $\pm$ 0.21-fold). Our observations demonstrated that kisspeptin, but not GnRH, was upregulated by E2 and that kisspeptin stimulates GnRH mRNA expression in primary cultures of whole fetal rat brain. Furthermore, GnRH and kisspeptin stimulate their own neurons to produce GnRH or kisspeptin, respectively.

Keywords

GnRH, kisspeptin, estradiol, hypothalamus

Introduction

Gonadotropin-releasing hormone (GnRH) has been defined as a crucial component of the hypothalamic–pituitary–gonadal (HPG) axis in maintaining reproductive function by stimulating synthesis and release of the pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). After the identification of humans and mice carrying mutations in the Gq protein–coupled kisspeptin receptor *Kiss1R* (formerly termed GPR54), deficits in fertility, puberty, and sex steroids with low gonadotropin levels revealed that kisspeptin and Kiss1R play a pivotal role in reproductive functions.[1-3] The importance of kisspeptin and Kiss1R were further characterized with the use of *Kiss1*- and *Kiss1R*-null mice,[4,5] and now it is generally agreed that hypothalamic kisspeptin directly acts at the level of GnRH neurons and induces GnRH release. This concept is based on previous observations that GnRH neurons express Kiss1R, and kisspeptin-induced LH secretion from the pituitary was blocked by a GnRH antagonist.[6] A number of studies have demonstrated that kisspeptin can induce gonadotropin secretion in mammals by acting through GnRH neurons.[7] In addition, kisspeptin expression in the hypothalamus is strongly involved in the negative feedback control of the HPG axis by sex steroids.[8,9]

Many studies on the role of kisspeptin in GnRH neurons have focused on the secretion of GnRH by measuring gonadotropin levels.[6,10] In addition, Novaira et al demonstrated the stimulatory effect of kisspeptin on GnRH messenger RNA (mRNA) expression and secretion in GnRH-secreting neuronal cell lines, GT1-7 and GN11.[11]

Although GT1-7 cells endogenously express Kiss1R, kisspeptin does not affect these cells, likely because the sensitivity of endogenous Kiss1R to kisspeptin is limited.[12] In our GT1-7 cells, kisspeptin demonstrably stimulated intracellular signaling cascades such as extracellular signal-regulated kinase (ERK) and cyclic adenosine monophosphate (cAMP)/protein

[1] Department of Obstetrics and Gynecology, Faculty of Medicine, Shimane University School of Medicine, Izumo, Shimane, Japan
[2] Department of Experimental Animals, Interdisciplinary Center for Science Research, Organization for Research, Shimane University, Izumo, Shimane, Japan

Corresponding Author:
Haruhiko Kanasaki, Department of Obstetrics and Gynecology, Faculty of Medicine, Shimane University School of Medicine, 89-1 Enya-cho, Izumo, Shimane 693-8501, Japan.
Email: kanasaki@med.shimane-u.ac.jp

kinase A (PKA) pathways but only when cells were transfected with Kiss1R. In this series of experiments, we showed that kisspeptin increased GnRH receptor expression with a concomitant increase in ERK and cAMP/PKA signaling pathways in Kiss1R-transfected GT1-7 cells.[12] GnRH-expressing cell lines are useful models to explore the mechanisms by which kisspeptin regulates GnRH expression. However, there is a possibility that some characteristics of GnRH neurons could be altered by producing transgenic mice or establishing GnRH-producing cell lines.

In the present study, we examined the regulation of GnRH and kisspeptin mRNA expression. To ascertain whether kisspeptin affects GnRH neurons and increases its production, primary cultures of fetal rat brain containing a variety of neuronal types, including those expressing GnRH and kisspeptin, were used. We showed that kisspeptin did not increase GnRH mRNA expression in GT1-7 cells, even when Kiss1R was overexpressed in these cells.

Materials and Methods

Materials

The following chemicals and reagents were obtained from the indicated sources: GIBCO fetal bovine serum (FBS; Invitrogen, Carlsbad, California); Dulbecco modified Eagle medium (DMEM), water-soluble β-estradiol (E2), GnRH, and penicillin-streptomycin (Sigma-Aldrich Co, St Louis, Missouri); kisspeptin-10 (AnaSpec, Fremont, California); and DNase I (Promega Co, Madison, Wisconsin).

Cell Culture and Transfection

GT1-7 cells,[13] kindly provided by Dr P. Mellon of the University of California (San Diego, California), were plated in 35-mm tissue culture dishes and incubated with high-glucose DMEM containing 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37°C under a humidified atmosphere of 5% CO_2 in air. After 24 hours, the culture medium was changed to high-glucose DMEM containing 1% heat-inactivated FBS and 1% penicillin-streptomycin and incubated without (control) or with the test reagents for the indicated periods. Cells were transiently transfected by electroporation with 2.0 μg/well of the human Kiss1R expression vector, which was generously provided by Dr Ursula Kaiser (Brigham and Women's Hospital and Harvard University). An empty vector (pCI-neo) served as a mock control.

Primary Culture of Neuronal Cells of Fetal Rat Brain

Six to 8 fetal rat brains were obtained from fetuses from a female rat at 16 to 18 days of gestation under deep sodium pentobarbital anesthesia. Whole brains from fetal rats were excised and minced before incubating in calcium- and magnesium-free Hanks' balanced salt solution (CMF-HBSS) containing 10 mg/mL trypsin and 2 mg/mL collagenase (Nitta Gelatin, Osaka, Japan) for 15 minutes at 37°C. Samples were then incubated in an identical solution containing 0.5 μg/mL DNase I (Boehringer-Mannheim, Mannheim, Germany) for 5 minutes at 37°C. After incubation in CMF-HBSS containing 5-mmol/L EDTA (Wako Pure Chemicals, Osaka, Japan) for 5 minutes at 37°C, the samples were washed with CMF-HBSS. Dispersed cells were then suspended in CMF-HBSS using a pipette, passed through a 70-μm nylon mesh (Becton Dickinson Labware, Franklin Lakes, New Jersey), and then collected by centrifugation. The pellet was suspended and 2 to 3×10^5 cells were cultured on a 35-mm petri dish in DMEM medium with 10% FBS and 1% penicillin-streptomycin until use. This protocol was approved by the committee of the Experimental Animal Center for Integrated Research in Shimane University.

Immunocytochemistry

The cells were fixed on coverslips by a 10-minute methanol treatment at −20°C. After 10 minutes of dehydration at 25°C, the cells were treated with 0.2% Triton X-100 in phosphate-buffered saline (PBS) for permeabilization. Nonspecific antibody binding was blocked by preincubation with 1% albumin, followed by an overnight incubation at 4°C with anti-GnRH 1 antibody (1:50 dilution; Santa Cruz Biotechnology Inc, Dallas, Texas) and an anti-kisspeptin antibody (1:500 dilution; Millipore, Billerica, Massachusetts). To visualize GnRH and kisspeptin, the cells were stained with secondary antibodies according to the manufacturer's instructions for the use of a Histofine SAB-PO (MULTI) Kit (Nichirei Bioscience Inc, Tokyo, Japan). The biotinylated secondary antibodies were coupled with streptavidin-biotinylated horseradish peroxidase, and the reaction was visualized using diaminobenzidine as the chromogen. Chemiluminescence images of the cells were obtained with an Olympus BX41 microscope (Olympus, Tokyo, Japan).

RNA Preparation, Reverse Transcription, and Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA from neuronal cells was extracted using TRIzol-S (Invitrogen) according to the manufacturer's instructions. To obtain complementary DNA (cDNA), 1.0 μg total RNA was reverse transcribed using an oligo-dT primer (Promega) and prepared using a First-Strand cDNA Synthesis Kit (Invitrogen) in reverse transcription buffer. The preparation was supplemented with 10 mmol/L dithiothreitol, 1 mmol/L of each deoxyribonucleoside triphosphate, and 200 U RNase inhibitor/human placenta ribonuclease inhibitor (Code No. 2310; Takara, Tokyo, Japan) in a final volume of 10 μL. The reaction was incubated at 37°C for 60 minutes. Quantification of GnRH and kisspeptin was obtained through real-time quantitative polymerase chain reaction (ABI Prism 7000; Perkin-Elmer Applied Biosystems, Foster City, California) following the manufacturer's protocol (User Bulletin No. 2) and utilizing a Universal Probe Library Probe and Fast Start Master Mix (Roche Diagnostics, Mannheim, Germany). Using specific primers for GnRH

(forward: 5′-ACTGTGTGTTTGGAAGGCTGC-3′ and reverse: 5′-TTCCAGAGCTCCTCGCAGATC-3′) and kisspeptin (forward: 5′-ATGATCTCGCTGGCTTCTTGG-3′ and reverse: 5′-GGTTCACCACAGGTGCCATTTT-3′), the simultaneous measurement of mRNA and glyceraldehyde-3-phosphate dehydrogenase permitted normalization of the amount of cDNA added per sample. For each set of primers, a no-template control was included. Thermal cycling conditions were as follows: 10-minute denaturation at 95°C, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. The crossing threshold was determined using PRISM 7000 software, and postamplification data were analyzed using the delta-delta CT method in Microsoft Excel.

Western Blot Analysis

GT1-7 cells were rinsed with PBS and lysed on ice with radioimmunoprecipitation assay buffer (PBS, 1% nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) containing 0.1 mg/mL phenylmethylsulfonyl fluoride, 30 mg/mL aprotinin, and 1 mmol/L sodium orthovanadate; scraped for 20 seconds; and centrifuged at 14 000*g* for 10 minutes at 4°C. Protein concentration in the cell lysates was measured using the Bradford method of protein quantitation. Denatured protein per well (10 μg) was separated on a 10% SDS–polyacrylamide gel electrophoresis gel according to standard protocols. Protein was transferred onto polyvinylidene difluoride (PVDF) membranes (Hybond-P PVDF, Amersham Biosciences, Little Chalfont, United Kingdom), which was blocked for 2 hours at room temperature in Blotto (5% milk in Tris-buffered saline). Membranes were incubated with phospho-ERK antibody (p-ERK; 1:250 dilution; Santa Cruz Biotechnology Inc) in Blotto overnight at 4°C and washed 3 times for 10 minutes per wash with Tris-buffered saline/1% Tween. Subsequent incubation with monoclonal horseradish peroxidase (HRP)-conjugated antibody was performed for 1 hour at room temperature in Blotto, and additional washes were performed appropriately. Following enhanced chemiluminescence detection (Amersham Biosciences), membranes were exposed onto X-ray film (Fujifilm, Tokyo, Japan). After strip washing (Restore buffer; Pierce Chemical Co, Rockford, Illinois), membranes were reprobed with ERK antibody (T-ERK; 1:10 000 dilution; Santa Cruz Biotechnology Inc) for 1 hour at room temperature and then incubated with HRP-conjugated secondary antibody before the procedure was continued as described earlier. Films were analyzed by densitometry, and the intensity of p-ERK was normalized to that of T-ERK to correct protein loading.

Statistical Analysis

All experiments were repeated independently at least 3 times, and each experiment was performed in duplicate for each experimental group. Data are expressed as means ± standard error of the mean. Statistical analysis was performed using 1-way analysis of variance, followed by Newman-Keuls multiple comparison test.

Results

Effect of Kisspeptin on GnRH mRNA Expression in GT1-7 Cells

GT1-7 cells are immortalized hypothalamic GnRH-producing neuronal cell lines that were developed by genetically targeted tumorigenesis.[13] These cells are used as a valuable model for the study of the function of GnRH neurons. In our lines of GT1-7 cells, GnRH mRNA expression was unchanged in the presence of kisspeptin at all time points after stimulation (mock; Figure 1A). Because we had already established that GT1-7 cells respond to kisspeptin only when the Kiss1R is abundantly expressed,[12] GT1-7 cells were transiently transfected with Kiss1R expression vectors. After the transfection of Kiss1R, however, kisspeptin failed to stimulate GnRH mRNA expression (Figure 1B). We confirmed that kisspeptin increased phosphorylation of ERK in Kiss1R-transfected GT1-7 cells (Figure 1D) but not in mock transfected GT1-7 cells (Figure 1C).

Expression of GnRH and Kisspeptin in Primary Cultures of Fetal Rat Brain

The cultured neuronal cells contained several types of cells. Immunostaining showed that GnRH- and kisspeptin-expressing cells were present in this culture (Figure 2).

Effect of E2 on GnRH and Kisspeptin mRNA Expression

First, we examined whether GnRH and kisspeptin, which are expressed by neurons in the fetal brain, are influenced by E2. Expression of GnRH mRNA was not significantly increased by culturing the cells for 48 hours in the presence of different concentrations of E2 (Figure 3A). However, 10 μmol/L E2, but not 1 μmol/L, significantly increased kisspeptin mRNA expression by 2.00 ± 0.23-fold compared to unstimulated cells (Figure 3B).

Expression of GnRH and Kisspeptin mRNA Is Mutually Regulated by GnRH and Kisspeptin

The previous in vivo studies support the hypothesis that kisspeptin controls gonadotropin secretion by stimulating GnRH neurons in the hypothalamus. Next, we examined the effect of kisspeptin on GnRH expression. Kisspeptin could significantly increase GnRH mRNA expression, determined at 48 hours after stimulation, with an increase of up to 1.51 ± 0.35-fold. Interestingly, GnRH itself increased GnRH mRNA expression by 1.84 ± 0.33-fold, which was statistically significant (Figure 4A). Kisspeptin mRNA expression in the primary cultures of fetal rat brain was stimulated by kisspeptin itself by 2.43 ± 0.40-fold. In addition, GnRH could increase kisspeptin mRNA expression by 1.45 ± 0.21-fold (Figure 4B).

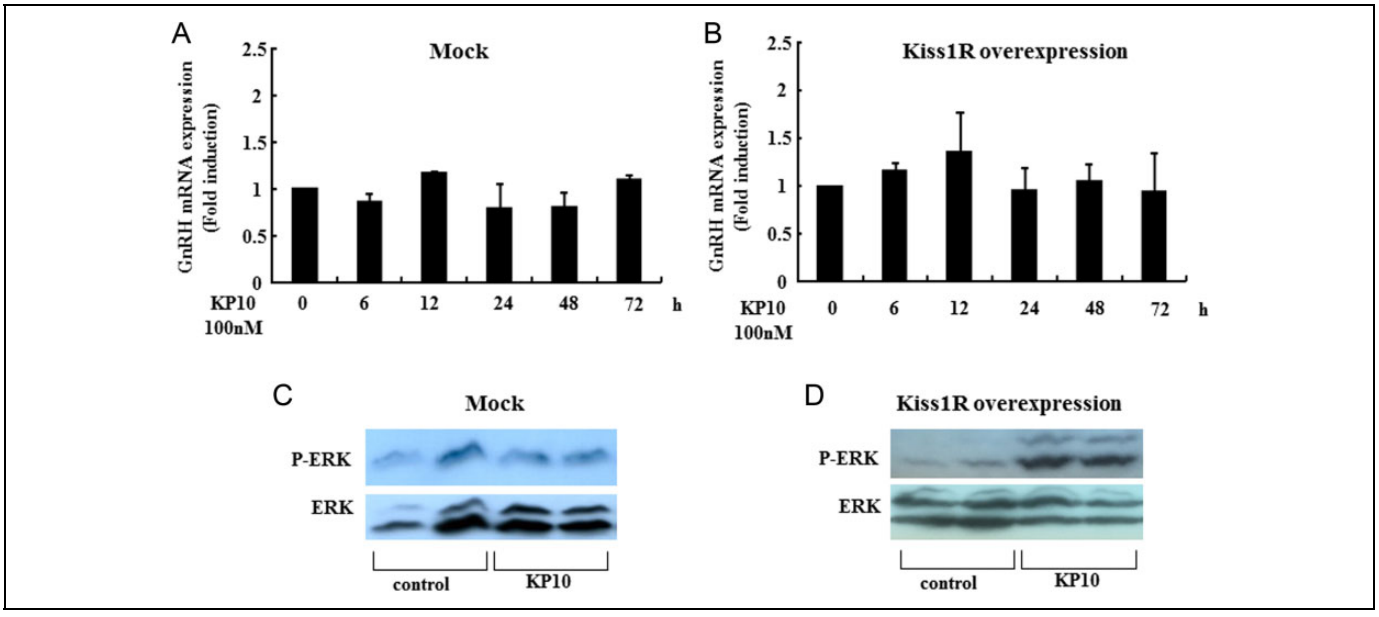

Figure 1. Effect of kisspeptin on GnRH mRNA expression in GT1-7 cells. GT1-7 cells were transfected without (mock; A) and with 2.0 μg of Kiss1R-expressing vectors (B). At 48 hours after transfection, cells were treated with 100 nmol/L kisspeptin (KP10) for indicated times. Then, GnRH mRNA levels were measured by quantitative real-time PCR after mRNA extraction and reverse transcription. Samples for each experimental group were run in duplicate and normalized to GAPDH mRNA levels as a housekeeping gene. Results are expressed as fold stimulation over the unstimulated group/control. Values are the means ± SEM of fold stimulation from independent experiments. To confirm the response of the cells, mock (C) or Kiss1R-transfected GT1-7 cells (D) were stimulated with 100 nmol/L kisspeptin for 10 minutes ERK phosphorylation was determined by Western blotting using phosphorylated-ERK (P-ERK) and total ERK(T-ERK) antibodies, as described in the Materials and Methods section. ERK indicates extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GnRH, gonadotropin-releasing hormone; mRNA, messenger RNA; PCR, polymerase chain reaction; SEM, standard error of the mean.

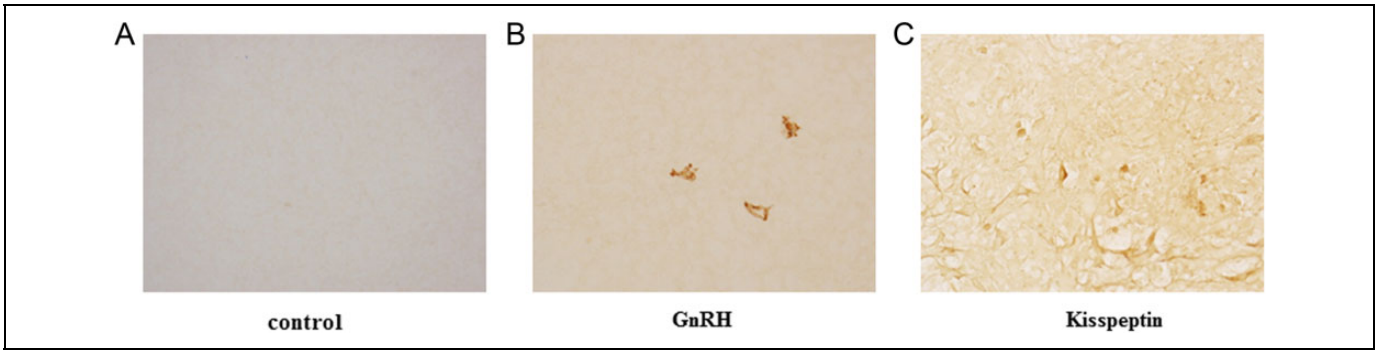

Figure 2. Staining of primary cultures of fetal rat neuronal cells for gonadotropin-releasing hormone (GnRH) and kisspeptin. Cultures of neuronal cells from fetal rats at 18 weeks of gestation were immunostained using anti-GnRH (B) and anti-kisspeptin (C) antibodies. The negative control is shown in (A; control). The colors were developed by streptavidin-biotinylated horseradish peroxidase. Magnification, ×20.

Discussion

Accumulating evidence supports the concept that kisspeptin signals act directly on GnRH neurons through Kiss1R to stimulate GnRH release, which subsequently stimulates the secretion of LH and FSH from the anterior pituitary.[14] Although GnRH cannot be measured in the peripheral circulation, much evidence shows that kisspeptin acts directly on GnRH neurons. The GnRH neurons express Kiss1R, and kisspeptin has been shown to cause depolarization of GnRH neurons.[15] Kisspeptin also stimulates the release of GnRH in hypothalamic explants.[16] However, these in vivo studies did not directly address whether kisspeptin could stimulate GnRH synthesis at the single-cell level. To determine the direct action of kisspeptin on GnRH-producing neurons, we first used the GnRH-producing cell line GT1-7. In our series of experiments, we could not discern any stimulatory effect of kisspeptin on GnRH mRNA expression in GT1-7 cells. Even in Kiss1R-overexpressing cells, kisspeptin failed to increase GnRH mRNA levels. Because intracellular signaling pathways such as ERK and cAMP/PKA were activated by kisspeptin when cells were transfected with Kiss1R, and because we observed that GnRH receptor expression in GT1-7 cells was increased by

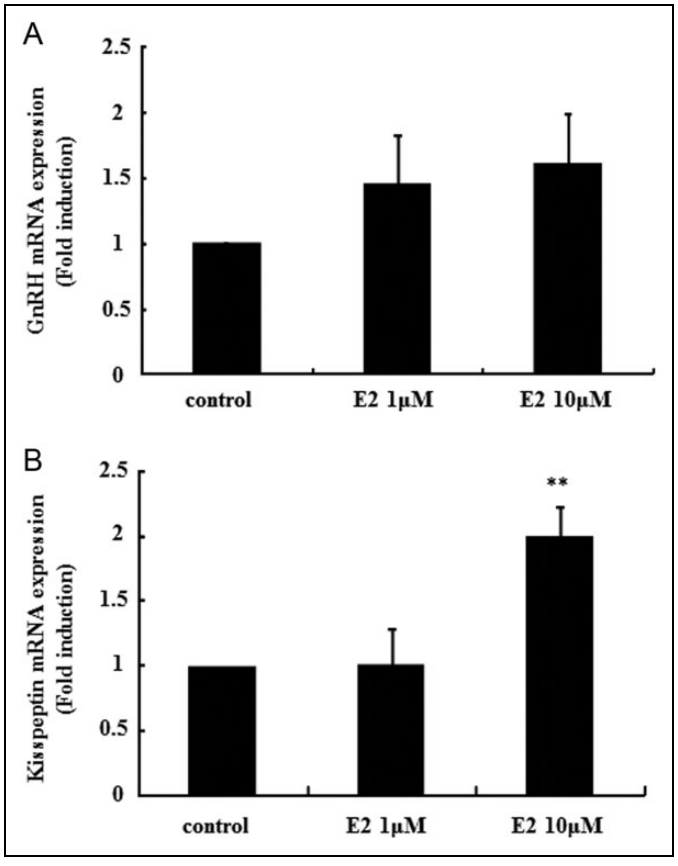

Figure 3. Effect of estradiol on GnRH and kisspeptin mRNA expression in neuronal cells from fetal rats. Primary cultures of fetal rat neuronal cells were cultured in the presence or absence (control) of 1 μmol/L or 10 μmol/L estradiol (E2) for 48 hours. Then, GnRH (A) and kisspeptin (B) mRNA levels were measured by quantitative real-time PCR after mRNA extraction and reverse transcription. Samples for each experimental group were run in duplicate and normalized to GAPDH mRNA levels as a housekeeping gene. Results are expressed as fold stimulation over the unstimulated group/control. Values are the means ± SEM of fold stimulation from independent experiments. $^{**}P < .01$ versus control. GnRH indicates gonadotropin-releasing hormone; mRNA, messenger RNA; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SEM, standard error of the mean.

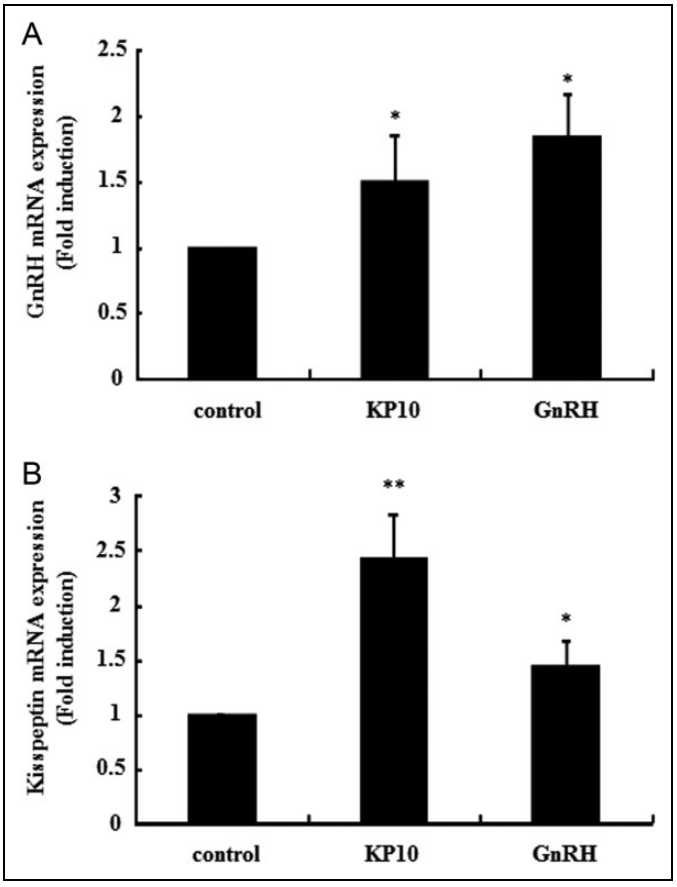

Figure 4. Effect of kisspeptin and GnRH on GnRH and kisspeptin mRNA expression in neuronal cells from fetal rats. Primary cultures of fetal rat neuronal cells were treated with kisspeptin (KP10; 100 nmol/L) and GnRH (100 nmol/L) for 48 hours. Then, GnRH (A) and kisspeptin (B) mRNA levels were measured by quantitative real-time PCR after mRNA extraction and reverse transcription. Samples for each experimental group were run in duplicate and normalized to GAPDH mRNA levels as a housekeeping gene. Results are expressed as fold stimulation over the unstimulated group/control. Values are the means ± SEM of fold stimulation from independent experiments. $^{**}P < .01$, $^{*}P < .05$ versus control. GnRH indicates gonadotropin-releasing hormone; mRNA, messenger RNA; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SEM, standard error of the mean.

kisspeptin,[12] it is unlikely that the loss of GnRH production in response to kisspeptin is responsible for the loss of Kiss1R function in GT1-7 cells. Because some studies have demonstrated a direct effect of kisspeptin on GnRH mRNA expression using GT1-7 cells,[11,17] these differences in GnRH synthesis in response to kisspeptin might be due to differences in GT1-7 cell lines or phenotypic changes resulting from multiple passages. The changes in the expression and/or sensitivity of Kiss1R in GT1-7 cells may be affected by culture conditions.

To clearly determine whether hypothalamic kisspeptin stimulates GnRH synthesis in GnRH neurons, we used primary cultures of fetal rat brain. These cells contained both GnRH and kisspeptin as expected, and we examined the effect of GnRH and kisspeptin on their mRNA expression. First, we examined the effect of E2 because it is one of the most important regulators of neuronal activity in the HPG axis. In our experiment, using primary cultures of rat brain, E2 failed to stimulate GnRH mRNA expression. Although a direct role of E2 in GnRH neuronal function has been disaffirmed by the lack of estrogen receptor immunoreactivity in GnRH neurons,[18] growing evidence suggests the presence of functional estrogen receptors in GnRH neurons.[19,20] Estradiol did not affect the GnRH-expressing cells from fetal brain; however, kisspeptin expression was clearly increased by E2 treatment. These observations demonstrated that kisspeptin, but not GnRH neurons, could be a target of E2 in neuronal cells in the fetal brain. Kisspeptin neurons exist in 2 different populations within the brain. The previous studies have provided evidence that estrogen-dependent induction of kisspeptin mRNA occurs in

the population of kisspeptin neurons within the anterioventricular nucleus (AVPV) and exerts estrogen-dependent positive feedback effects on the hypothalamus, which triggers preovulatory GnRH/LH surges in female animals.[21,22] Conversely, kisspeptin neurons within the arcuate nucleus (ARC), which coexpress neurokinin B and dynorphin (ie, KNDy neurons), have been determined to be an estrogen-negative feedback target because estrogen treatment decreases kisspeptin mRNA levels in kisspeptin neurons in this population.[21,23] We are presently unsure how kisspeptin neurons exist in these 2 populations in the fetal brain, and it still unknown whether estrogen feedback mechanisms appropriately work within the hypothalamic neuronal cells from fetal rat brain. Nevertheless, our results show that kisspeptin, but not GnRH-expressing neurons, in fetal rat brain responds to E2 and increases kisspeptin expression.

Our main purpose of this study was to clarify whether kisspeptin increases GnRH mRNA expression. Expression of GnRH mRNA was indeed increased by treatment with kisspeptin in the primary cultures of fetal rat brain containing GnRH-producing neurons, suggesting that kisspeptin can stimulate GnRH synthesis in GnRH-expressing neurons. We assumed that if kisspeptin is a principle regulator of GnRH, then kisspeptin would drastically increase GnRH expression. However, unexpectedly, kisspeptin increased GnRH mRNA expression only up to about 1.5-fold.

Keen et al measured the release of kisspeptin in the stalk-median eminence by the microdialysis method in female rhesus monkeys and demonstrated that kisspeptin release in the region was clearly pulsatile with an interpulse interval of about 60 minutes.[24] In our present study, we directly added kisspeptin to cell culture dishes to examine whether, if mode of stimulation was altered, kisspeptin might evoke more dramatic changes in GnRH mRNA. We also could not exclude the possibility that the original characteristics, including sensitivity of Kiss1R, may have been changed by mechanical separation of the neuronal cells or by cell culture conditions. Two distinct kisspeptin neurons with different phenotypes exist in 2 distinct regions of the brain (AVPV and ARC), then E2 would be expected to have different effects on these neurons. The proportion of kisspeptin neurons in these 2 distinct populations might be the cause of the limited increase in kisspeptin expression, because E2 treatment increases GnRH synthesis in 1 cell population and prevents it in the other, as described earlier. In another case, kisspeptin may originally have little ability to stimulate GnRH mRNA expression, despite playing a crucial role in gonadotropin release via GnRH. It is also well known that energy-metabolic condition and therefore nutritional condition is important in reproductive regulation. Our cell models using fetal brain might be not sufficient to maintain the reproductive regulation system, such as the kisspeptin-GnRH axis. In the present experiments, GnRH mRNA expression was also increased by GnRH itself to a degree similar to that produced by kisspeptin stimulation. The previous reports demonstrated that GnRH neurons respond to GnRH and have an autocrine interaction with GnRH. Autocrine GnRH stimulation generates cell survival and proliferative signals[25] or modulates GnRH release.[26]

Numerous studies on the regulatory roles of kisspeptin have focused on gonadal steroids, which were implicated by association with feedback mechanisms of the HPG axis. Interestingly, we found that kisspeptin mRNA expression in primary cultures of fetal rat brain was increased by kisspeptin itself as well as by GnRH. These observations suggest the possibility that GnRH neurons reversibly interact with kisspeptin neurons and that autocrine interactions within kisspeptin neurons could exist in the HPG axis. Although it has been shown that kisspeptin neurons project to the cell bodies of GnRH neurons in the optic area and to the median eminence, which are closed to GnRH nerve terminals,[27,28] it is still unknown whether kisspeptin neurons are affected by GnRH neurons through their nerve terminals. In addition, it is unclear whether kisspeptin neurons possess Kiss1Rs on their surface. However, if kisspeptin could stimulate its own expression, kisspeptin expression might be maintained by autocrine regulation within the population of kisspeptin neurons. In addition to the sex steroids, kisspeptin expression was reported to be implicated with several hypothalamic hormones such as leptin,[29] α-melanocyte-stimulating hormone,[30] γ-aminobutyric acid (GABA),[31] and corticotropin-releasing factor.[32] More work is needed to explore the detailed mechanisms of kisspeptin regulation.

In summary, we have found that kisspeptin, which is under the control of E2, stimulates GnRH mRNA expression in primary cultures of fetal rat neuronal cells. The GnRH also has the ability to stimulate kisspeptin in these cell populations. These neuropeptides may possess reciprocal action within the neuronal cells. Further analysis is warranted.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

References

1. de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci U S A*. 2003;100(19):10972-10976.
2. Funes S, Hedrick JA, Vassileva G, et al. The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. *Biochem Biophys Res Commun*. 2003;312(4):1357-1363.
3. Seminara SB, Messager S, Chatzidaki EE, et al. The GPR54 gene as a regulator of puberty. *N Engl J Med*. 2003;349(17):1614-1627.
4. Kauffman AS, Park JH, McPhie-Lalmansingh AA, et al. The kisspeptin receptor GPR54 is required for sexual differentiation of the brain and behavior. *J Neurosci*. 2007;27(33):8826-8835.
5. Lapatto R, Pallais JC, Zhang D, et al. Kiss1-/- mice exhibit more variable hypogonadism than Gpr54-/- mice. *Endocrinology*. 2007;148(10):4927-4936.

6. Irwig MS, Fraley GS, Smith JT, et al. Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. *Neuroendocrinology*. 2004; 80(4):264-272.
7. Kauffman AS. Coming of age in the kisspeptin era: sex differences, development, and puberty. *Mol Cell Endocrinol*. 2010; 324(1-2):51-63.
8. Smith JT, Clay CM, Caraty A, Clarke IJ. KiSS-1 messenger ribonucleic acid expression in the hypothalamus of the ewe is regulated by sex steroids and season. *Endocrinology*. 2007;148(3): 1150-1157.
9. Smith JT, Li Q, Pereira A, Clarke IJ. Kisspeptin neurons in the ovine arcuate nucleus and preoptic area are involved in the preovulatory luteinizing hormone surge. *Endocrinology*. 2009; 150(12):5530-5538.
10. Navarro VM, Castellano JM, Fernández-Fernández R, et al. Characterization of the potent luteinizing hormone-releasing activity of KiSS-1 peptide, the natural ligand of GPR54. *Endocrinology*. 2005;146(1):156-163.
11. Novaira HJ, Ng Y, Wolfe A, Radovick S. Kisspeptin increases GnRH mRNA expression and secretion in GnRH secreting neuronal cell lines. *Mol Cell Endocrinol*. 2009;311(1-2):126-134.
12. Sukhbaatar U, Kanasaki H, Mijiddorj T, Oride A, Miyazaki K. Kisspeptin induces expression of gonadotropin-releasing hormone receptor in GnRH-producing GT1-7 cells overexpressing G protein-coupled receptor 54. *Gen Comp Endocrinol*. 2013;194: 94-101.
13. Mellon PL1, Windle JJ, Goldsmith PC, Padula CA, Roberts JL, Weiner RI. Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. *Neuron*. 1990;5(1):1-10.
14. Skorupskaite K, George JT, Anderson RA. The kisspeptin-GnRH pathway in human reproductive health and disease. *Hum Reprod Update*. 2014;20(4):485-500.
15. Han SK, Gottsch ML, Lee KJ, et al. Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. *J Neurosci*. 2005;25(49):11349-11356.
16. Thompson EL, Patterson M, Murphy KG, et al. Central and peripheral administration of kisspeptin-10 stimulates the hypothalamic-pituitary-gonadal axis. *J Neuroendocrinol*. 2004; 16(10):850-858.
17. Terasaka T, Otsuka F, Tsukamoto N, et al. Mutual interaction of kisspeptin, estrogen and bone morphogenetic protein-4 activity in GnRH regulation by GT1-7 cells. *Mol Cell Endocrinol*. 2013; 381(1-2):8-15.
18. Shivers BD, Harlan RE, Morrell JI, Pfaff DW. Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones. *Nature*. 1983;304(5924):345-347.
19. Hrabovszky E, Steinhauser A, Barabás K, et al., Estrogen receptor-beta immunoreactivity in luteinizing hormone-releasing hormone neurons of the rat brain. *Endocrinology*. 2001;142(7):3261-3264.
20. Hrabovszky E, Kalló I, Szlávik N, Keller E, Merchenthaler I, Liposits Z. Gonadotropin-releasing hormone neurons express estrogen receptor-beta. *J Clin Endocrinol Metab*. 2007;92(7): 2827-2830.
21. Kinoshita M1, Tsukamura H, Adachi S, et al. Involvement of central metastin in the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. *Endocrinology*. 2005;146(10):4431-4436.
22. Clarkson J, d'Anglemont de Tassigny X, Moreno AS, Colledge WH, Herbison AE. Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. *J Neurosci*. 2008;28(35): 8691-8697.
23. Adachi S, Yamada S, Takatsu Y, et al. Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats. *J Reprod Dev*. 2007;53(2):367-378.
24. Keen KL, Wegner FH, Bloom SR, Ghatei MA, Terasawa E. An increase in kisspeptin-54 release occurs with the pubertal increase in luteinizing hormone-releasing hormone-1 release in the stalk-median eminence of female rhesus monkeys in vivo. *Endocrinology*. 2008;149(8):4151-4157.
25. Neithardt A, Farshori MP, Shah FB, Catt KJ, Shah BH. Dependence of GnRH-induced phosphorylation of CREB and BAD on EGF receptor transactivation in GT1-7 neuronal cells. *J Cell Physiol*. 2006;208(3):586-593.
26. Krsmanovic LZ, Martinez-Fuentes AJ, Arora KK, et al. Autocrine regulation of gonadotropin-releasing hormone secretion in cultured hypothalamic neurons. *Endocrinology*. 1999;140(3): 1423-1431.
27. Clarkson J, Herbison AE. Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. *Endocrinology*. 2006;147(12):5817-5825.
28. Uenoyama Y, Inoue N, Pheng V, et al. Ultrastructural evidence of kisspeptin-gonadotrophin-releasing hormone (GnRH) interaction in the median eminence of female rats: implication of axo-axonal regulation of GnRH release. *J Neuroendocrinol*. 2011;23(10): 863-870.
29. Smith JT, Acohido BV, Clifton DK, Steiner RA. KiSS-1 neurones are direct targets for leptin in the ob/ob mouse. *J Neuroendocrinol*. 2006;18(4):298-303.
30. Backholer K, Smith J, Clarke IJ. Melanocortins may stimulate reproduction by activating orexin neurons in the dorsomedial hypothalamus and kisspeptin neurons in the preoptic area of the ewe. *Endocrinology*. 2009;150(12):5488-5497.
31. Martin C, Navarro VM, Simavli S, et al. Leptin-responsive GABAergic neurons regulate fertility through pathways that result in reduced kisspeptinergic tone. *J Neurosci*. 2014;34(17): 6047-6056.
32. Kinsey-Jones JS, Li XF, Knox AM, et al. Down-regulation of hypothalamic kisspeptin and its receptor, Kiss1r, mRNA expression is associated with stress-induced suppression of luteinising hormone secretion in the female rat. *J Neuroendocrinol*. 2009; 21(1):20-29.