Action of Neurotensin, CRH, and RFRP-3 in E2-induced Negative Feedback Control: Studies Using a Mouse ARC Hypothalamic Cell Model

Tuvshintugs Tumurbaatar[a], Haruhiko Kanasaki[a], Aki Oride[a], Tomomi Hara[a], Hiroe Okada[a], Kazuyoshi Tsutsui[b], and Satoru Kyo[a]

[a]Department of Obstetrics and Gynecology, Shimane University School of Medicine, Izumo 693-8501, Japan

[b]Laboratory of Integrative Brain Science, Department of Biology, Waseda University, and Center for Medical Life Science of Waseda University, Tokyo 162-8480, Japan

Short title: NT, CRH, and RFRP-3 action in negative feedback

Key words; kisspeptin, neurotensin, CRH, RFRP-3, hypothalamus, ARC

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (H.K.).

Corresponding Author: Haruhiko Kanasaki, MD, PhD
Department of Obstetrics and Gynecology, School of Medicine, Shimane University
89-1 Enya Cho, Izumo, Shimane 693-8501, Japan

Tel.: +81-853-20-2268; Fax: +81-853-20-2264
Email: kanasaki@med.shimane-u.ac.jp

Abstract

The recently established immortalized hypothalamic cell model mHypoA-55 possesses characteristics similar to those of Kiss-1 neurons in the arcuate nucleus (ARC) region of the hypothalamus. Here, we show that Kiss-1 gene expression in these cells was downregulated by 17β-estradiol (E2) under certain conditions. Both neurotensin (NT) and corticotropin-releasing hormone (CRH) were expressed in these cells and upregulated by E2. Stimulation of mHypoA-55 cells with NT and CRH significantly decreased Kiss-1 mRNA expression. A mammalian gonadotropin-inhibitory hormone homolog, RFamide-related peptide-3 (RFRP-3), was also found to be expressed in mHypoA-55 cells, and RFRP-3 expression in these cells was increased by exogenous melatonin stimulation. E2 stimulation also upregulated RFRP-3 expression in these cells. Stimulation of mHypoA-55 cells with RFRP-3 significantly increased the expression of NT and CRH. Furthermore, melatonin stimulation resulted in the increase of both NT and CRH mRNA expression in mHypoA-55 cells. On the other hand, in experiments using mHypoA-50 cells, which were originally derived from hypothalamic neurons in the anteroventral periventricular nucleus, Kiss-1 gene expression was upregulated by both NT and CRH, although E2 increased both NT and CRH expression, similarly to the mHypoA-55 cells.

Our observations using the hypothalamic ARC cell model mHypoA-55 suggest that NT and CRH have inhibitory effects on Kiss-1 gene expression under the influence of E2 in association with RFRP-3 expression. Thus, these neuropeptides might be involved in E2-induced negative feedback mechanisms.

Introduction

Neurons in the hypothalamus, especially those that secrete gonadotropin-releasing hormone (GnRH), serve as the final pathway by which the brain regulates gonadotropin secretion [1]. After the discovery of inactivating mutations in the kisspeptin receptor (Kiss1R) in families of patients with hypogonadotropic hypogonadism [2, 3], it is generally agreed that hypothalamic Kiss-1 neurons, which project to GnRH neurons, control GnRH release through Kiss1R expressed by the GnRH neurons. Therefore, Kiss-1 neurons in the hypothalamus have been defined as being positioned at the highest level in the control of the hypothalamic-pituitary-gonadal (HPG) axis. These neurons have been shown to be located in two different regions of the hypothalamus: the arcuate nucleus (ARC) and the anteroventral periventricular (AVPV) region in mammals [4]. Although studies have demonstrated a bimodal effect of 17β-estradiol (E2) on the hypothalamus, having both positive and negative feedback effects on GnRH neurons that do not express estrogen receptor α (ERα) [5], it is now postulated that Kiss-1 neurons within the ARC are the center for negative feedback by E2, whereas those in the AVPV are defined as the center of positive feedback. These conclusions are based on the observations that Kiss-1 expression in the ARC is inhibited by E2, whereas AVPV Kiss-1 expression is stimulated by E2 [6].

Kiss-1 neurons might also play a role as a GnRH pulse generator. Previous studies recorded multiunit activity that correlates with pulsatile secretion of pituitary luteinizing hormone (LH) in a variety of animals [7, 8]. A neuronal population within the ARC, referred to as KNDy neurons, express Kiss-1, neurokinin B (NKB), and dynorphin (Dyn). Kiss-1–expressing KNDy neurons have been shown to generate

synchronized oscillatory patterns of activity by receiving autosynaptic excitatory and inhibitory input from NKB and Dyn [9, 10].

Although Kiss-1 neurons in the ARC and AVPV express and release kisspeptin, these two populations possess distinct characteristics. Owing to the heterogeneous nature and complexity of the hypothalamus, investigation into the molecular events involved in E2-induced feedback control in unique neuronal cell populations has been difficult. Recently, a hypothalamic cell model, mHypoA-55, was established from microdissected ARC primary cultures of female-adult-derived murine hypothalamus, which exhibits endogenous Kiss-1 expression as well as ERα and ERβ. mHypoA-55 cells also express NKB and Dyn, suggesting that they represent ARC kisspeptin neurons [11]. On the other hand, mHypoA-50 cells, which were established from microdissected AVPV primary cultures, are also known as a Kiss-1–expressing cell model that possesses ERα and ERβ. However, although mHypoA-50 cells express tyrosine hydroxylase, they do not express NKB and Dyn, supporting their identity as putative AVPV kisspeptin neurons [11]. Notably, these cell models exhibit different responses to E2 in the regulation of Kiss-1 gene expression. E2 induces an increase in Kiss-1 mRNA expression in mHypoA-50 cells, but it downregulates Kiss-1 mRNA expression in mHypoA-55 cells under certain experimental conditions [11]. Therefore, the molecular events involved in differential E2-mediated regulation, which supposedly occurs in the AVPV and/or ARC region of the hypothalamus, could be examined using these cell models.

In this study, we aimed to examine E2-induced negative feedback mechanisms using ARC hypothalamic mHypoA-55 cells. We focused on the action of neurotensin (NT) and corticotropin-releasing hormone (CRH). NT was first isolated from bovine

hypothalamus in recognition of its potent vascular effects [12]. In mammals, NT is abundantly expressed in the central nervous system, with particularly high expression in the hypothalamus, including the AVPV and ARC regions [13, 14], and has been implicated in regulating a diverse repertoire of physiological functions and motivated behaviors including feeding, locomotor activity, social behavior, and reproduction [15-17]. CRH is a most important physiological ACTH-releasing factor and is involved in the stress response. CRH is mainly produced within the paraventricular nucleus of the hypothalamus and is released at the median eminence from the neurosecretory terminals of these neurons [18]. In addition, CRH neurons are found in many other regions in the brain, including the olfactory bulb, medial preoptic nucleus, and middle hypothalamus [19].

We found that NT and CRH might participate in the E2-induced negative feedback regulation of the HPG axis in association with RFamide-related peptide-3 (RFRP-3), a mammalian gonadotropin-inhibitory hormone (GnIH) homolog.

Materials and Methods

Materials

The following chemicals and reagents were obtained from the indicated sources: GIBCO fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA); Dulbecco's modified Eagle's medium (DMEM), water-soluble E2, penicillin-streptomycin, and melatonin (Sigma-Aldrich Co., St. Louis, MO); and NT and CRH (Life Technologies Japan., Ltd., Tokyo, Japan). RFRP-3 was synthesized with an automated solid-phase peptide synthesizer (PSSM-8; Shimadzu, Kyoto, Japan) and purified by reversed phase high-performance liquid chromatography.

Cell culture

mHypoA-50 and mHypoA-55 cells were purchased from CEDARLANE (Ontario, Canada). The embryonic rat hypothalamic cell line R8 (rHypoE-8) was purchased from COSMO BIO CO., LTD. (Tokyo, Japan). Cells 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 h, cells were used for each experiment. When cells were stimulated by E2, cells were cultured with E2 in phenol red–free DMEM supplemented with 1% charcoal-stripped FBS (Gemini Bio-Products, West Sacramento, CA). When stimulated with the indicated neuropeptides, cells were incubated without (control) or with the test reagents in high-glucose DMEM containing 1% heat-inactivated FBS and 1% penicillin-streptomycin for the indicated time periods.

Western blot analysis

Cell extracts were lysed on ice with RIPA buffer (phosphate-buffered saline

[PBS], 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing 0.1 mg/mL phenylmethylsulfonyl fluoride, 30 mg/mL aprotinin, and 1 mM sodium orthovanadate, scraped for 20 s, and centrifuged at 14,000 × *g* for 10 min at 4°C. Protein concentration in the cell lysates was measured using the Bradford method. Denatured protein (10 µg per well) was resolved in a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel according to standard protocols. Protein was transferred onto polyvinylidene difluoride membranes (Hybond-P PVDF, Amersham Biosciences, Little Chalfont, UK), which were blocked for 2 h at room temperature in Blotto (5% milk in Tris-buffered saline). Membranes were incubated with anti-kisspeptin antibody (1:500 dilution; Abcam, Cambridge, UK), anti-NT antibody (1:200 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX), or anti-CRH antibody (1:200 dilution; Santa Cruz Biotechnology, Inc.) in Blotto overnight at 4°C and washed 3 times for 10 min per wash with Tris-buffered saline/1% Tween. Subsequent incubation with horseradish peroxidase (HRP)-conjugated antibodies was performed for 1 h at room temperature in Blotto, and additional washes were performed appropriately. Following enhanced chemiluminescence detection (Amersham Biosciences), membranes were exposed to X-ray film (Fujifilm, Tokyo, Japan). Fetal rat brain tissue was used as positive control, and this experimental protocol was approved by the animal care and use committee of the Experimental Animal Center for Integrated Research at Shimane University (IZ27-82).

RNA preparation, reverse transcription, PCR, and quantitative real-time PCR

Total RNA from stimulated cells was extracted using TRIzol-LS (Invitrogen) according to the manufacturer's instructions. To obtain cDNA, 1.0 µg total RNA was

reverse transcribed using an oligo-dT primer (Promega, Madison, WI) and prepared using a First-Strand cDNA Synthesis Kit (Invitrogen) in reverse transcription (RT) buffer. The preparation was supplemented with 10 mM dithiothreitol, 1 mM of each dNTP, 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 min. For the detection of Kiss-1, NT, and CRH mRNAs, after PCR amplification using primers for rat Kiss-1 (forward: 5′-ATGATCTCGCTGGCTTCTTGG-3′ and reverse: 5′-GGTTCACCACAGGTGCCATTTT-3′), NT (forward: 5′-GTGTGGACCTGCTTGTCAGA-3′ and reverse: 5′-TCATGCATGTCTCCTGCTTC-3′), CRH (forward: 5′-ATCCGCATGGGTGAAGAATACT-3′ and reverse: 5′-TGGAAGGTGAGATCCAGAGAGA-3′) and RFRP-3 (forward: 5′-CAGGGACCAGGAGCCATTTC and reverse: 5′-CTCCTCTCCTCGTTCGCTTTC-3′), amplicons were electrophoresed in agarose gels and visualized with ethidium bromide staining. Quantification of Kiss-1, NT, and CRH mRNA was obtained through quantitative real-time PCR (ABI Prism 7000; Perkin-Elmer Applied Biosystems, Foster City, CA) following the manufacturer's protocol (User Bulletin No. 2) and utilizing Universal ProbeLibrary Probes and FastStart Master Mix (Roche Diagnostics, Mannheim, Germany). Using specific primers for rat Kiss-1, NT, CRH, and RFRP-3, the simultaneous measurement of mRNA and GAPDH 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 min denaturation at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Reactions were followed by

melting curve analysis (55–95°C). To determine PCR efficiency, a 10-fold serial dilution of cDNA was performed as previously described [20]. PCR conditions were optimized to generate >95% PCR efficiency and only those reactions with between 95% and 105% efficiency were included in subsequent analyses. Relative differences in cDNA concentration between baseline and experimental conditions were then calculated using the comparative threshold cycle (Ct) method [21]. Briefly, for each sample, a ΔCt was calculated to normalize to the internal control using the following equation: ΔCt = ΔCt(gene) – Ct(GAPDH). To obtain differences between experimental and control conditions, ΔΔCt was calculated as ΔCt(sample) – ΔCt(control). Relative mRMA levels were then calculated using the following equation: fold difference = $2^{\Delta\Delta Ct}$.

Statistical analysis

All experiments were repeated independently at least three times. Each experiment in each experimental group was performed using duplicate samples. When we determined the mRNA expression, two samples were assayed in duplicate. Six averages from three independent experiments were statistically analyzed. Data are expressed as mean ± SEM values. Statistical analysis was performed using Student's *t* test or one-way analysis of variance (ANOVA) with Bonferroni's post hoc test, as appropriate. $P < 0.05$ was considered statistically significant.

Results

mHypoA-55 cells possess characteristics similar to those of kisspeptin neurons in the ARC region

We used the recently established cell line mHypoA-55 as a model of hypothalamic Kiss-1–expressing cells in the ARC region. As previously demonstrated, mHypoA-55 cells express both Kiss-1 mRNA and the kisspeptin protein. mHypoA-50 cells, which are derived from Kiss-1–expressing cells in the AVPV region, also express both Kiss-1 mRNA and kisspeptin. Although both cell models express kisspeptin, these cells are distinct. mHypoA-55 cells express both Tac2 (which encodes NKB) and Dyn genes, but mHypoA-50 cells do not express these genes (Fig. 1A). The responses to E2 in these cell models were also distinct. A previous study demonstrated that Kiss-1 gene expression was increased by E2 at 24 h in mHypoA-50 cells, whereas it was reduced by E2 at 4 h in mHypoA-55 cells [11]. Similar to the previous results, Kiss-1 mRNA expression in mHypoA-55 cells was not increased by E2; rather, it was significantly reduced at 4 h after the addition of E2 and returned to the control level at 24 h ($F(2,6) = 20.38$, $P = 0.0021$) (Fig. 1B). In contrast, E2 treatment significantly increased Kiss-1 mRNA expression up to 3.03 ± 0.54-fold at 24 h in the mHypoA-50 AVPV cell model ($F(2,15) = 7.025$, $P = 0.0070$) (Fig. 1C).

Effect of NT on Kiss-1 mRNA expression in mHypoA-55 cells

The hypothalamic model mHypoA-55 cells exhibit a heterogeneous character; these cells express NT mRNA and its protein product in addition to Kiss-1, NKB, and Dyn. NT expression was also observed in mHypoA-50 cells (Fig. 2A). NT expression within mHypoA-55 cells was upregulated in the presence of E2. When mHypoA-55

cells were stimulated with E2 for 24 h, NT expression within these cells was significantly increased 1.71 ± 0.19-fold ($F(2,6) = 8.16$, $P = 0.019$). A significant increase in NT mRNA expression was not observed by stimulation with E2 for 4 h (the time at which Kiss-1 mRNA expression was significantly reduced by E2 treatment in mHypoA-55 cells) (Fig. 2B). When mHypoA-55 cells were stimulated by NT, Kiss-1 mRNA expression was significantly reduced ($F(3,8) = 9.92$, $P = 0.0045$). At 10 nM NT, Kiss-1 mRNA expression was reduced to almost half that of untreated cells (Fig. 2C).

Effect of E2 on CRH expression in mHypoA-55 cells

CRH is a hypothalamic neuropeptide that controls the hypothalamic-pituitary-adrenal axis. Accumulating evidence suggests the importance of this peptide in reproductive function [22, 23]. CRH mRNA was detected by RT-PCR from the extracts of mHypoA-55 ARC and mHypoA-50 AVPV cells. CRH protein was also expressed in these cells (Fig. 3A). Next, we examined the role of CRH in mHypoA-55 cells. As observed in NT experiments, CRH expression in mHypoA-55 was also influenced by E2. By 24 h, but not 4 h of incubation with E2, CRH mRNA levels in mHypo-A55 cells were significantly increased by 5.89 ± 1.87-fold ($F(2,6) = 5.93$, $P = 0.038$) (Fig. 3B). When mHypoA-55 cells were stimulated by CRH, Kiss-1 mRNA expression in these cells was significantly reduced to 0.62 ± 0.17-fold at 100 nM versus the untreated control cells ($F(3,8) = 8.64$, $P = 0.0069$) (Fig. 3C), which was similar to the effect of NT.

RFRP-3 expression in mHypoA-55 cells and the effect of melatonin

The avian homolog of RFRP-3, GnIH, was first identified as neuropeptide that

inhibits gonadotropin release in birds [24]. We found that the RFRP-3 gene is expressed in mHypoA-55 cells (Fig. 4A). Similar to the phenomenon observed in birds [25], 10 μM melatonin stimulation increased RFRP-3 mRNA expression 1.93 ± 0.39-fold in mHypoA-55 cells ($F(3,12) = 4.69$, $P = 0.022$) (Fig. 4B). In addition, RFRP-3 expression in mHypoA-55 cells was slightly but significantly upregulated by E2 stimulation: 1.55 ± 0.19-fold compared with unstimulated cells ($t(1.94) = 2.74$, $P = 0.017$) (Fig. 4C).

Effect of RFRP-3 stimulation on the expression of NT and CRH in mHypoA-55 cells

Next, we examined how RFRP-3 affects the expression of NT and CRH genes in mHypoA-55 cells. mHypoA-55 cells were simulated with increasing concentrations of RFRP-3 and cultured for 24 h, and then NT and CRH mRNA expression was examined. NT mRNA expression was significantly increased by RFRP-3 1.94 ± 0.24-fold at 100 nM ($F(6,12) = 6.35$, $P = 0.0080$) (Fig. 5A). CRH mRNA expression was also stimulated by RFRP-3. At all tested concentrations (10 nM, 100 nM, and 1 μM), RFRP-3 significantly increased CRH mRNA expression ($F(3,12) = 3.93$, $P = 0.036$), and was increased 2.39 ± 0.72-fold at 10 nM (Fig. 5B). Furthermore, we found that melatonin, which had the ability to stimulate RFRP-3 expression, increased the NT and CRH gene expression by 1.55 ± 0.21-fold and 2.96 ± 0.54-fold, respectively, both of which were statistically significant (NT: $t(2.13) = 3.96$, $P = 0.0083$; CRH: $t(2.13) = 3.63$, $P = 0.011$) (Fig. 6A and B).

Effect of NT and CRH on the expression of the Kiss-1 gene in the AVPV cell model, mHypoA-50

As shown above, similar to the mHypoA-55 ARC Kiss-1–expressing cell model, AVPV model mHypoA-50 cells also express NT and CRH. Lastly, we examined how these neuropeptides affect Kiss-1 gene expression in mHypoA-50 cells. When mHypoA-50 cells were stimulated with E2, NT expression within these cells was significantly increased 1.71 ± 0.19-fold at 24 h ($F(2,6) = 12.87$, $P = 0.0068$) (Fig. 7A). This observation was similar to those observed in ARC model mHypoA-55 cells. However, in contrast with NT's inhibition of Kiss-1 gene expression in mHypoA-55 cells, compared with the unstimulated control, 10 nM NT increased Kiss-1 mRNA expression 3.63 ± 1.45-fold in mHypoA-50 cells ($t(2.13) = 2.19$, $P = 0.047$) (Fig. 7B). Similarly, CRH mRNA expression in mHypoA-50 cells was increased by E2 stimulation, as was observed in mHypoA-55 cells. By incubation with E2, CRH mRNA levels in mHypo-A50 were significantly increased by 3.2 ± 0.38-fold at 4 h and 3.74 ± 0.93-fold at 24 h ($F(2,6) = 6.22$, $P = 0.035$) (Fig. 7C). In mHypo-A50 cells, Kiss-1 mRNA expression was significantly increased by stimulating the cells with CRH (1.75 ± 0.29-fold) ($t(1.86) = 2.61$, $P = 0.016$) (Fig. 7D). This CRH effect in mHypoA-50 cells was completely the opposite of that observed in mHypoA-55 cells.

Discussion

Studies on the cellular mechanisms involving the E2-induced positive and negative feedback mechanisms are somewhat difficult because the hypothalamus consists of a complex, heterogeneous array of neuronal phenotypes. Development of the hypothalamic Kiss-1–expressing cell models mHypoA-55 and mHypo-A50 has enabled the examination of the local mechanisms of E2-induced feedback control that occur in specific regions of the hypothalamus in rodents. At present, accumulating evidence supports the concept that the hypothalamic Kiss-1 neurons in the ARC region generate a pulsatile mode of GnRH secretion and mediate the negative feedback action of sex steroids [6, 26]. Conversely, Kiss-1 neurons in the AVPV region of the hypothalamus are considered to control the surge mode of GnRH secretion evoked by the positive feedback action of E2 [27, 28]. These conclusions are based on the observations that Kiss-1 expression in the ARC is inhibited by E2, whereas AVPV Kiss-1 expression is stimulated by E2 [6].

In this study, we mainly utilized mHypo-A55 cells as a model for the ARC region of the hypothalamus and examined the possible mechanisms of the E2-induced negative feedback control. As reported previously [11], mHypoA-55 cells have characteristics similar to those of Kiss-1 neurons in the ARC region. These cells coexpress NKB and Dyn, and significant repression of Kiss-1 expression was observed under certain conditions (4 h after E2 stimulation) [11]. Similar to the phenomenon observed in the previous study, Kiss-1 expression was not increased by E2; rather, significant repression of Kiss-1 was observed at 4 h in our mHypoA-55 cells. In contrast, E2 significantly increased Kiss-1 expression in AVPV model mHypoA-50 cells. Therefore, we used mHypoA-55 cells as a model to study the E2-induced negative

feedback mechanisms.

NT and CRH have been reported to have some influence on the HPG axis. Several lines of evidence implicate NT-producing cells as a target for the action of E2 to trigger the GnRH/LH surge. E2 induces the expression of the NT gene in AVPV lesions in rats [29, 30]. In addition, it was reported that administration of NT directly into the rat medial preoptic area evoked LH secretion, whereas antiserum directed against NT delivered into this region blocked the LH surge [31, 32]. These observations suggest NT has some effect on E2-induced positive feedback mechanisms. On the other hand, a number of studies suggest CRH as a prime candidate for stress-induced suppression of the GnRH pulse generator [23, 33], although CRH can stimulate LH secretion and increases GnRH pulse amplitude in sheep [34, 35]. CRH expression in the hypothalamus has also been reported to be increased after administration of E2 in ovariectomized monkeys [36].

In the hypothalamic ARC cell model, mHypoA-55, we confirmed that NT and CRH mRNA expression was upregulated by E2, and these observations were consistent with previous reports that E2 could positively control the expression of these neuropeptides. Interestingly, NT significantly reduced Kiss-1 gene expression in the mHypoA-55 cells. Furthermore, similar to the effect of NT, exogenous CRH administration reduced Kiss-1 gene expression in mHypoA-55 cells. This is quite similar to the phenomenon observed in a previous *in vivo* study, which showed that CRH could decrease Kiss-1 expression in the medial preoptic area and ARC in rats [37]. Our observation that NT and CRH expression was upregulated by E2 implies that NT and CRH could be involved in E2-induced negative feedback regulation of the pulsatile

GnRH release in the hypothalamus. In contrast, exogenous NT and CRH administration differently affected the AVPV cell model mHypoA-50; both NT and CRH increased Kiss-1 gene expression in mHypoA-50 cells, although these peptides were upregulated by E2 in mHypoA-50 cells. These observations suggest that NT and CRH have a completely different role in Kiss-1 neurons in the AVPV region. In addition, these results reconfirm that the Kiss-1–expressing cells exhibit different characteristics in each cell model. These results concerning the distinct action of NT and CRH in mHypoA-55 and mHypoA-50 imply that the E2-mediated increase in NT and CRH expression stimulates the production of Kiss-1 in the AVPV region, ultimately initiating the surge in GnRH secretion from GnRH neurons, as previous studies have suggested. Indeed, a previous study reported that close apposition of NT- and Kiss-1–expressing cells was observed, although NT and Kiss-1 mRNAs were not coexpressed in the mouse AVPV neurons [38]. In addition, it was reported that kisspeptin neurons in both the ARC and AVPV express the GnRH receptor and close apposition of GnRH immunoreactive fibers was observed on some Kiss-1 neurons in both regions [39]. Our current observations imply that NT and CRH are involved in the E2-induced positive and negative feedback mechanism by differentially changing the Kiss-1 gene expression in the AVPV and ARC regions of the hypothalamus. However, we should note that our observations do not completely support this hypothesis because an increasing effect of E2 on NT and CRH mRNA expression was not observed at 4 h of E2 treatment, the time at which Kiss-1 mRNA expression was significantly reduced by E2 treatment in mHypoA-55 cells. Hypothetically, if E2-induced NT or CRH expression was involved in the reduction of Kiss-1 gene expression in mHypoA-55 cells, E2-induced reduction of Kiss-1 gene expression should have occurred at 24 h after stimulation. Our present

data do not directly address the mechanism(s) underlying the time lag of the E2-stimulated increase in NT and CRH mRNA expression or the E2-mediated reduction of Kiss-1 gene expression in mHypoA-55 cells, both of which will likely be investigated in future studies.

In this study, we observed that mHypoA-55 cells express RFRP-3, a mammalian homolog of GnIH. In mice, cell bodies of GnIH neurons are located in the dorsomedial hypothalamic nucleus [40], and its neuronal fibers are in close proximity to GnRH and kisspeptin neurons that express the GnIH receptor [41]. Because mHypoA-55 cells express the RFRP-3 gene, it is plausible that mHypoA-55 cells are derived from GnIH-expressing neurons in the ARC region of hypothalamus. RFRP-3 mRNA expression within mHypoA-55 cells was upregulated by melatonin stimulation and this observation was similar to that observed in GnIH neurons in birds [25], suggesting that RFRP-3–expressing mHypoA-55 cells have characteristics similar to those of GnIH neurons in birds. Interestingly, RFRP-3 has the ability to stimulate the expression of NT and CRH, both of which could repress Kiss-1 gene expression. These observations imply that NT and CRH as well as RFRP-3 might participate in the negative feedback control, supposedly by repressing Kiss-1 gene expression. GnIH neurons have been shown to express ERα and respond to E2 [40]. Because we observed that RFRP-3 expression in mHypoA-55 cells was upregulated by E2, we can speculate on the possible role of RFRP-3 in E2-induced negative feedback mechanisms. There are several published reports that indicate the possible involvement of GnIH in the mechanism of E2-induced positive feedback control based on the observation that E2 suppresses GnIH gene expression [41-43]. RFRP-3 might be the gatekeeper of the E2-induced negative feedback control, but we speculate that RFRP-3–associated

E2-independent mechanisms might be involved in the suppression of the Kiss-1 gene through NT and CRH. It has been shown that environmental stress can increase the expression of GnIH and suppress reproduction in vertebrates [44]. Furthermore, glucocorticoids can directly control GnIH expression [45]. Because melatonin could regulate the expression of RFRP-3, the photoperiodic milieu or eye activity may be involved in the reduction of Kiss-1 gene expression by increasing NT or CRH. Our results showing that melatonin stimulation increased the expression of NT and CRH mRNAs support this hypothesis. However, we should keep in mind that the effect of RFRP-3 or melatonin differs depending on the type of experimental model. GnIH and RFRP-3 have been shown to inhibit GnRH neuron activity and gonadotropin release in several seasonal animals (sheep, hamster, and quail) and non-seasonal species such as rat and mouse. In contrast, no evidence of RFRP-3 directly modulating LH secretion was observed in ewes [46, 47]. Furthermore, recent studies using male Syrian and Siberian hamsters demonstrated the stimulatory effects of RFRP-3 on the reproductive axis [48, 49]. In the female Syrian hamster, RFRP-3 works alongside Kiss-1 neurons to maintain proper synchronization of reproductive activity with the time of day, the stage of the estrous cycle, and the seasonal changes in photoperiods [50]. RFRP-3 controls seasonal reproduction [51] and also controls feeding and reproductive behaviors [52].

Using a Kiss-1–expressing cell model for the ARC region of the hypothalamus, we propose the possible involvement of E2-induced or RFRP-3–associated NT and CRH expression in the regulation of Kiss-1 gene expression in the ARC hypothalamic region that governs negative feedback mechanisms in the HPG axis. We used immortalized hypothalamic cell models, and it should be noted that clonal cell lines come with inherent limitations. These cells do not represent the intact brain, with necessary

connections and architecture. It is still unclear whether the phenomenon observed in this study occurs *in vivo*. NT, CRH, and RFRP-3 are expressed in the hypothalamus and their neuronal fibers indeed exist in the ARC region in each experimental model [39, 53, 54]. However, coexpression of these peptides in ARC Kiss-1 neurons is uncertain. Nevertheless, we were able to dissect the molecular signaling events controlling Kiss-1/NT/CRH and RFRP-3 neurons using this cell model. Furthermore, these models may allow for the investigation of paracrine and autocrine regulation of these neuropeptides.

We summarized our current results in Figure 8. In this study, we used an ARC hypothalamic cell model in which Kiss-1 gene expression was repressed by E2 under certain conditions (4 h after E2 stimulation). CRH and NT expression was upregulated by E2 and we found that NT and CRH negatively regulated Kiss-1 gene expression in these cells. Because RFRP-3, which was regulated by melatonin or E2, could induce the expression of NT and CRH in these cells, RFRP-3 might be involved in the suppression of Kiss-1 gene expression in the ARC region of the hypothalamus. Although NT and CRH were similarly increased by E2, these two peptides increased the Kiss-1 gene expression in an AVPV hypothalamic cell model. Our findings might provide clues regarding the feedback mechanism of E2 in the hypothalamus.

References

1. Crowley WF, Jr., Filicori M, Spratt DI, Santoro NF. The physiology of gonadotropin-releasing hormone (GnRH) secretion in men and women. Recent Prog Horm Res 1985; 41:473-531.
2. 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; 100:10972-10976.
3. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Jr., Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, et al. The GPR54 gene as a regulator of puberty. N Engl J Med 2003; 349:1614-1627.
4. Clarkson J, d'Anglemont de Tassigny X, Colledge WH, Caraty A, Herbison AE. Distribution of kisspeptin neurones in the adult female mouse brain. J Neuroendocrinol 2009; 21:673-682.
5. Radovick S, Ticknor CM, Nakayama Y, Notides AC, Rahman A, Weintraub BD, Cutler GB, Jr., Wondisford FE. Evidence for direct estrogen regulation of the human gonadotropin-releasing hormone gene. J Clin Invest 1991; 88:1649-1655.
6. Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA. Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 2005; 146:3686-3692.
7. Wilson RC, Kesner JS, Kaufman JM, Uemura T, Akema T, Knobil E. Central electrophysiologic correlates of pulsatile luteinizing hormone secretion in the rhesus monkey. Neuroendocrinology 1984; 39:256-260.
8. Mori Y, Nishihara M, Tanaka T, Shimizu T, Yamaguchi M, Takeuchi Y, Hoshino K. Chronic recording of electrophysiological manifestation of the hypothalamic gonadotropin-releasing hormone pulse generator activity in the goat. Neuroendocrinology 1991; 53:392-395.
9. Wakabayashi Y, Nakada T, Murata K, Ohkura S, Mogi K, Navarro VM, Clifton DK, Mori Y, Tsukamura H, Maeda K, Steiner RA, Okamura H. Neurokinin B and dynorphin A in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat. J Neurosci 2010; 30:3124-3132.
10. Navarro VM, Gottsch ML, Chavkin C, Okamura H, Clifton DK, Steiner RA. Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. J Neurosci 2009; 29:11859-11866.
11. Treen AK, Luo V, Chalmers JA, Dalvi PS, Tran D, Ye W, Kim GL, Friedman Z,

Belsham DD. Divergent Regulation of ER and Kiss Genes by 17beta-Estradiol in Hypothalamic ARC Versus AVPV Models. Mol Endocrinol 2016; 30:217-233.

12. Carraway R, Leeman SE. The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami. J Biol Chem 1973; 248:6854-6861.

13. Carraway R, Leeman SE. Characterization of radioimmunoassayable neurotensin in the rat. Its differential distribution in the central nervous system, small intestine, and stomach. J Biol Chem 1976; 251:7045-7052.

14. Rostene WH, Alexander MJ. Neurotensin and neuroendocrine regulation. Front Neuroendocrinol 1997; 18:115-173.

15. Cape EG, Manns ID, Alonso A, Beaudet A, Jones BE. Neurotensin-induced bursting of cholinergic basal forebrain neurons promotes gamma and theta cortical activity together with waking and paradoxical sleep. J Neurosci 2000; 20:8452-8461.

16. Merullo DP, Cordes MA, Stevenson SA, Riters LV. Neurotensin immunolabeling relates to sexually-motivated song and other social behaviors in male European starlings (Sturnus vulgaris). Behav Brain Res 2015; 282:133-143.

17. Brown JA, Bugescu R, Mayer TA, Gata-Garcia A, Kurt G, Woodworth HL, Leinninger GM. Loss of Action via Neurotensin-Leptin Receptor Neurons Disrupts Leptin and Ghrelin-Mediated Control of Energy Balance. Endocrinology 2017; 158:1271-1288.

18. Vale W, Spiess J, Rivier C, Rivier J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science 1981; 213:1394-1397.

19. Peng J, Long B, Yuan J, Peng X, Ni H, Li X, Gong H, Luo Q, Li A. A Quantitative Analysis of the Distribution of CRH Neurons in Whole Mouse Brain. Front Neuroanat 2017; 11:63.

20. Wong ML, Medrano JF. Real-time PCR for mRNA quantitation. Biotechniques 2005; 39:75-85.

21. Bustin SA, Benes V, Nolan T, Pfaffl MW. Quantitative real-time RT-PCR--a perspective. J Mol Endocrinol 2005; 34:597-601.

22. Rivier C, Vale W. Influence of corticotropin-releasing factor on reproductive functions in the rat. Endocrinology 1984; 114:914-921.

23. Cates PS, Li XF, O'Byrne KT. The influence of 17beta-oestradiol on corticotrophin-releasing hormone induced suppression of luteinising hormone pulses and the role of CRH in hypoglycaemic stress-induced suppression of pulsatile LH secretion in the female rat. Stress 2004; 7:113-118.

24. Tsutsui K, Saigoh E, Ukena K, Teranishi H, Fujisawa Y, Kikuchi M, Ishii S, Sharp

PJ. A novel avian hypothalamic peptide inhibiting gonadotropin release. Biochem Biophys Res Commun 2000; 275:661-667.

25. Ubuka T, Bentley GE, Ukena K, Wingfield JC, Tsutsui K. Melatonin induces the expression of gonadotropin-inhibitory hormone in the avian brain. Proc Natl Acad Sci U S A 2005; 102:3052-3057.

26. Ohkura S, Takase K, Matsuyama S, Mogi K, Ichimaru T, Wakabayashi Y, Uenoyama Y, Mori Y, Steiner RA, Tsukamura H, Maeda KI, Okamura H. Gonadotrophin-releasing hormone pulse generator activity in the hypothalamus of the goat. J Neuroendocrinol 2009; 21:813-821.

27. Inoue N, Sasagawa K, Ikai K, Sasaki Y, Tomikawa J, Oishi S, Fujii N, Uenoyama Y, Ohmori Y, Yamamoto N, Hondo E, Maeda K, et al. Kisspeptin neurons mediate reflex ovulation in the musk shrew (Suncus murinus). Proc Natl Acad Sci U S A 2011; 108:17527-17532.

28. 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; 28:8691-8697.

29. Alexander MJ, Dobner PR, Miller MA, Bullock BP, Dorsa DM, Leeman SE. Estrogen induces neurotensin/neuromedin N messenger ribonucleic acid in a preoptic nucleus essential for the preovulatory surge of luteinizing hormone in the rat. Endocrinology 1989; 125:2111-2117.

30. Smith MJ, Wise PM. Neurotensin gene expression increases during proestrus in the rostral medial preoptic nucleus: potential for direct communication with gonadotropin-releasing hormone neurons. Endocrinology 2001; 142:3006-3013.

31. Akema T, Praputpittaya C, Kimura F. Effects of preoptic microinjection of neurotensin on luteinizing hormone secretion in unanesthetized ovariectomized rats with or without estrogen priming. Neuroendocrinology 1987; 46:345-349.

32. Ferris CF, Pan JX, Singer EA, Boyd ND, Carraway RE, Leeman SE. Stimulation of luteinizing hormone release after stereotaxic microinjection of neurotensin into the medial preoptic area of rats. Neuroendocrinology 1984; 38:145-151.

33. Li XF, Bowe JE, Kinsey-Jones JS, Brain SD, Lightman SL, O'Byrne KT. Differential role of corticotrophin-releasing factor receptor types 1 and 2 in stress-induced suppression of pulsatile luteinising hormone secretion in the female rat. J Neuroendocrinol 2006; 18:602-610.

34. Tilbrook AJ, Canny BJ, Stewart BJ, Serapiglia MD, Clarke IJ. Central administration of corticotrophin releasing hormone but not arginine vasopressin

stimulates the secretion of luteinizing hormone in rams in the presence and absence of testosterone. J Endocrinol 1999; 162:301-311.

35. Caraty A, Miller DW, Delaleu B, Martin GB. Stimulation of LH secretion in sheep by central administration of corticotrophin-releasing hormone. J Reprod Fertil 1997; 111:249-257.

36. Kerdelhue B, Jones GS, Gordon K, Seltman H, Lenoir V, Melik Parsadaniantz S, Williams RF, Hodgen GD. Activation of the hypothalamo-anterior pituitary corticotropin-releasing hormone, adrenocorticotropin hormone and beta-endorphin systems during the estradiol 17 beta-induced plasma LH surge in the ovariectomized monkey. J Neurosci Res 1995; 42:228-235.

37. Kinsey-Jones JS, Li XF, Knox AM, Wilkinson ES, Zhu XL, Chaudhary AA, Milligan SR, Lightman SL, O'Byrne KT. 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; 21:20-29.

38. Dungan Lemko HM, Naderi R, Adjan V, Jennes LH, Navarro VM, Clifton DK, Steiner RA. Interactions between neurotensin and GnRH neurons in the positive feedback control of GnRH/LH secretion in the mouse. Am J Physiol Endocrinol Metab 2010; 298:E80-88.

39. Takumi K, Iijima N, Higo S, Ozawa H. Immunohistochemical analysis of the colocalization of corticotropin-releasing hormone receptor and glucocorticoid receptor in kisspeptin neurons in the hypothalamus of female rats. Neurosci Lett 2012; 531:40-45.

40. Kriegsfeld LJ, Mei DF, Bentley GE, Ubuka T, Mason AO, Inoue K, Ukena K, Tsutsui K, Silver R. Identification and characterization of a gonadotropin-inhibitory system in the brains of mammals. Proc Natl Acad Sci U S A 2006; 103:2410-2415.

41. Ubuka T, Son YL, Tsutsui K. Molecular, cellular, morphological, physiological and behavioral aspects of gonadotropin-inhibitory hormone. Gen Comp Endocrinol 2016; 227:27-50.

42. Molnar CS, Kallo I, Liposits Z, Hrabovszky E. Estradiol down-regulates RF-amide-related peptide (RFRP) expression in the mouse hypothalamus. Endocrinology 2011; 152:1684-1690.

43. Salehi MS, Jafarzadeh Shirazi MR, Zamiri MJ, Pazhoohi F, Namavar MR, Niazi A, Ramezani A, Tanideh N, Tamadon A, Zarei A. Hypothalamic Expression of KiSS1 and RFamide-related Peptide-3 mRNAs during The Estrous Cycle of Rats. Int J Fertil Steril 2013; 6:304-309.

44. Chand D, Lovejoy DA. Stress and reproduction: controversies and challenges. Gen

Comp Endocrinol 2011; 171:253-257.
45. Son YL, Ubuka T, Narihiro M, Fukuda Y, Hasunuma I, Yamamoto K, Belsham DD, Tsutsui K. Molecular basis for the activation of gonadotropin-inhibitory hormone gene transcription by corticosterone. Endocrinology 2014; 155:1817-1826.
46. Caraty A, Blomenrohr M, Vogel GM, Lomet D, Briant C, Beltramo M. RF9 powerfully stimulates gonadotrophin secretion in the ewe: evidence for a seasonal threshold of sensitivity. J Neuroendocrinol 2012; 24:725-736.
47. Decourt C, Anger K, Robert V, Lomet D, Bartzen-Sprauer J, Caraty A, Dufourny L, Anderson G, Beltramo M. No Evidence That RFamide-Related Peptide 3 Directly Modulates LH Secretion in the Ewe. Endocrinology 2016; 157:1566-1575.
48. Ancel C, Bentsen AH, Sebert ME, Tena-Sempere M, Mikkelsen JD, Simonneaux V. Stimulatory effect of RFRP-3 on the gonadotrophic axis in the male Syrian hamster: the exception proves the rule. Endocrinology 2012; 153:1352-1363.
49. Ubuka T, Inoue K, Fukuda Y, Mizuno T, Ukena K, Kriegsfeld LJ, Tsutsui K. Identification, expression, and physiological functions of Siberian hamster gonadotropin-inhibitory hormone. Endocrinology 2012; 153:373-385.
50. Henningsen JB, Ancel C, Mikkelsen JD, Gauer F, Simonneaux V. Roles of RFRP-3 in the Daily and Seasonal Regulation of Reproductive Activity in Female Syrian Hamsters. Endocrinology 2017; 158:652-663.
51. Henningsen JB, Gauer F, Simonneaux V. RFRP Neurons - The Doorway to Understanding Seasonal Reproduction in Mammals. Front Endocrinol (Lausanne) 2016; 7:36.
52. Tsutsui K, Ubuka T. GnIH Control of Feeding and Reproductive Behaviors. Front Endocrinol (Lausanne) 2016; 7:170.
53. Woodworth HL, Brown JA, Batchelor HM, Bugescu R, Leinninger GM. Determination of neurotensin projections to the ventral tegmental area in mice. Neuropeptides 2018; 68:57-74.
54. Rizwan MZ, Poling MC, Corr M, Cornes PA, Augustine RA, Quennell JH, Kauffman AS, Anderson GM. RFamide-related peptide-3 receptor gene expression in GnRH and kisspeptin neurons and GnRH-dependent mechanism of action. Endocrinology 2012; 153:3770-3779.

Figure Legends

Figure 1

Hypothalamic mHypoA-55 and mHypoA-50 cell characteristics and the effect of E2 on Kiss-1 mRNA expression.

(A) Cell lysates (10 μg) from mHypoA-55 cells and mHypoA-50 cells were analyzed by SDS-PAGE followed by immunoblotting and incubation with antibodies against kisspeptin. The bands were visualized using HRP-conjugated secondary antibody. (B) RT-PCR analysis of Kiss-1, NKB (Tac2), and Dyn gene expression in mHypoA-55 and mHypoA-50 cells. Total RNA was prepared and RT-PCR was carried out for 40 cycles using NKB- (Tac2) and Dyn-specific primers. PCR products were resolved in a 1.5% agarose gel and visualized with ethidium bromide staining. mHypoA-55 cells (B) and mHypoA-50 cells (C) were stimulated with 100 nM E2 for 4 and 24 h, after which mRNA was extracted and reverse transcribed. Kiss-1 mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold induction over unstimulated cells and presented as mean ± SEM values of three independent experiments, each performed with duplicate samples. $^{**}P < 0.01$ vs. control. Statistical significance was determined by one-way ANOVA with Bonferroni's post hoc test.

Figure 2

Effect of E2 on NT mRNA expression and the effect of NT on Kiss-1 mRNA expression in mHypoA-55 cells.

(A) NT mRNA and its protein product were expressed in mHypoA-55 cells. Total RNA from mHypoA-55 and mHypoA-50 cells was prepared and RT-PCR was carried out for

40 cycles using an NT-specific primer. PCR products were resolved in a 1.5% agarose gel and visualized with ethidium bromide staining. Cell lysates (10 μg) from mHypoA-55 cells and mHypoA-50 cells were analyzed by SDS-PAGE followed by immunoblotting and incubation with antibodies against NT. The bands were visualized using HRP-conjugated secondary antibody. (B) mHypoA-55 cells were stimulated with 100 nM E2 for 4 and 24 h, after which mRNA was extracted and reverse transcribed. NT mRNA levels were measured by quantitative real-time PCR. (C) mHypoA-55 cells were stimulated with indicated concentrations of NT for 24 h, after which mRNA was extracted and reverse transcribed. Kiss-1 mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold induction over unstimulated cells and presented as mean ± SEM values of three independent experiments, each performed with duplicate samples. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control. Statistical significance was determined by one-way ANOVA with Bonferroni's post hoc test.

Figure 3

Effect of E2 on CRH mRNA expression and the effect of CRH on Kiss-1 mRNA expression in mHypoA-55 cells.

(A) Total RNA from mHypoA-55 and mHypoA-50 cells was prepared and RT-PCR was carried out for 40 cycles using a CRH-specific primer. PCR products were resolved in a 1.5% agarose gel and visualized with ethidium bromide staining. Cell lysates (10 μg) from mHypoA-55 cells and mHypoA-50 cells were analyzed by SDS-PAGE followed by immunoblotting and incubation with antibodies against CRH. The bands were visualized using HRP-conjugated secondary antibody. (B) mHypoA-55 cells were

stimulated with 100 nM E2 for 4 and 24 h, after which mRNA was extracted and reverse transcribed. CRH mRNA levels were measured by quantitative real-time PCR. (C) mHypoA-55 cells were stimulated with indicated concentrations of CRH for 24 h, after which mRNA was extracted and reverse transcribed. Kiss-1 mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold induction over unstimulated cells and presented as mean ± SEM values of three independent experiments, each performed with duplicate samples. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control. Statistical significance was determined by one-way ANOVA with Bonferroni's post hoc test.

Figure 4

RFRP-3 expression and the effect of melatonin or E2 on its gene expression in mHypoA-55 cells.

(A) Total RNA from mHypoA-55 cells was prepared and RT-PCR was carried out for 40 cycles using an RFRP-3–specific primer. PCR products were resolved in a 1.5% agarose gel and visualized with ethidium bromide staining. (B) mHypoA-50 cells were stimulated with indicated concentrations of melatonin for 24 h, after which mRNA was extracted and reverse transcribed. RFRP-3 mRNA levels were measured by quantitative real-time PCR. (C) mHypoA-55 cells were stimulated with 100 nM E2 for 24 h, after which mRNA was extracted and reverse transcribed. RFRP-3 mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold induction over unstimulated cells and presented as mean ± SEM values of three independent experiments, each performed with duplicate samples. $^{*}P < 0.05$ vs. control. Statistical significance was determined by Student's *t* test or one-way ANOVA with Bonferroni's

post hoc test.

Figure 5

Effect of RFRP-3 on the expression of NT and CRH mRNAs in mHypoA-55 cells. mHypoA-50 cells were stimulated with indicated concentrations of RFRP-3 for 24 h, after which mRNA was extracted and reverse transcribed. NT (A) and CRH (B) mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold induction over unstimulated cells and presented as mean ± SEM values of three independent experiments, each performed with duplicate samples. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control. Statistical significance was determined by one-way ANOVA with Bonferroni's post hoc test.

Figure 6

Effect of melatonin on the expression of NT and CRH mRNAs in mHypoA-55 cells. mHypoA-50 cells were stimulated with 10 nM melatonin for 24 h, after which mRNA was extracted and reverse transcribed. NT (A) and CRH (B) mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold induction over unstimulated cells and presented as mean ± SEM values of three independent experiments, each performed with duplicate samples. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control. Statistical significance was determined by Student's *t* test.

Figure 7

Effect of E2 on NT and CRH mRNA expression and the effect of these peptides on

Kiss-1 mRNA expression in the AVPV model mHypoA-50 cells.

The AVPV hypothalamic cell model mHypoA-50 was stimulated with 100 nM E2 for 4 and 24 h, after which mRNA was extracted and reverse transcribed. NT (A) and CRH (C) mRNA levels were measured by quantitative real-time PCR. mHypoA-50 cells were stimulated with 10 nM NT (B) and 100 nM CRH (D) for 24 h, after which mRNA was extracted and reverse transcribed. Kiss-1 mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold induction over unstimulated cells and presented as mean ± SEM values of three independent experiments, each performed with duplicate samples. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control. Statistical significance was determined by Student's *t* test or one-way ANOVA with Bonferroni's post hoc test.

Figure 8

Schematic summary of the possible involvement of NT and CRH in the negative feedback regulation by E2.

In mHypoA-55 AVPV Kiss-1–expressing cells, CRH and NT expression was increased by E2, and these peptides in turn negatively regulated Kiss-1 gene expression. Melatonin and E2 increased the expression of RFRP-3 in these cells, and RFRP-3 increased the expression of NT and CRH.