General and Comparative Endocrinology 270 (2019) 60–66

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Effect of pituitary adenylate cyclase-activating polypeptide (PACAP) in the regulation of hypothalamic kisspeptin expression☆

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ARTICLE INFO

Keywords:
PACAP
Kisspepton
HPG axis
Hypothalamus

ABSTRACT

Pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptor are broadly distributed in the brain, and PACAP is known to work as a multifunctional peptide. However, it is still largely unknown how PACAP affects the hypothalamic-pituitary-gonadal (HPG) axis. In this study, we examined the effect of PACAP on hypothalamic kisspeptin expression, a known regulator of gonadotropin-releasing hormone. We used two hypothalamic cell models, mHypoA-50 and mHypoA-55, which were originated from kisspeptin-expressing neuron in anterioventral periventricular nucleus and arcuate nucleus regions in the hypothalamus, respectively. Expression of Kiss-1 gene, which encodes kisspeptin, was significantly increased by PACAP stimulation in both mHypoA-50 and mHypoA-55 cells, by up to 2.69 ± 0.93-fold and 4.89 ± 1.13-fold, respectively. PACAP6-38, a PACAP receptor antagonist did not antagonize the action of PACAP on Kiss-1 gene expression but increased Kiss-1 gene by itself in these cells. PACAP-induced Kiss-1 gene expression in both mHypoA-50 and mHypoA-55 cells was almost completely prevented in the presence of H89, a protein kinase A inhibitor. PACAP was expressed in both these hypothalamic cell models and its expression was up-regulated by estradiol in mHypoA-50 cells but not in mHypoA-55 cells. Stimulation of mHypoA-50 and mHypoA-55 cells with PACAP increased the expression levels of corticotropin-releasing hormone and neurotensin, both of which could modulate HPG axis. Our present observations suggest that hypothalamic PACAP might modulate the HPG axis by directly or indirectly modulating Kiss-1 gene expression.

1. Introduction

Reproductive functions are precisely regulated by the hypothalamic-pituitary-gonadal (HPG) axis, and hypothalamic gonadotropin-releasing hormone (GnRH) has previously been defined as the primary regulator of the HPG axis. GnRH controls the release of pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), by changing the frequency and amplitude of their pulsatile release during the reproductive cycle (Crowley et al., 1985). LH and FSH cooperatively affect the gonads and regulate gametogenesis and steroid genesis (Gharib et al., 1990). Following the discovery of the mutation in the gene that encodes the receptor for kisspeptin (Kiss1 receptor:Kiss1R) (de Roux et al., 2003; Seminara et al., 2003), it was revealed that kisspeptin neurons in the hypothalamus control GnRH neurons through the Kiss1R as well as the pulsatile release of GnRH to the portal circulation (Maggi et al., 2016). Therefore, now it is generally considered that hypothalamic kisspeptin neurons have the most important role in the HPG axis.

Pituitary adenylate cyclase-activating polypeptide (PACAP) was first isolated from ovine hypothalamus extracts that could stimulate adenylate cyclase activity in anterior pituitary cells (Miyata et al., 1989). PACAP-containing neurons are distributed not only in the hypothalamus, but are broadly expressed in various brain regions including cerebral cortex, amygdala, hippocampus, and cerebral cortex (Gonzalez et al., 1998). PACAP and its receptor are expressed not only in the central nervous system (CNS), but also in the various tissues of peripheral organs where PACAP exerts various effects and has a role as a multifunctional peptide (Vaudry et al., 2009). Although PACAP produced from hypothalamus has a variety of functions, it can act as a hypophysiotropic hormone and stimulates the release of anterior pituitary hormones (Miyata et al., 1989; Okada et al., 2007).

Accumulating evidence suggests that PACAP has some effects in the

☆ 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 (to H. K.).

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https://doi.org/10.1016/j.ygcen.2018.10.006
Received 29 May 2018; Received in revised form 20 September 2018; Accepted 9 October 2018
Available online 11 October 2018

regulation of reproductive hormones involved in the HPG axis. A number of studies have implicated PACAP in the pubertal process by alteration of the activity of GnRH neurons (Choi et al., 2000; Szabo et al., 2002). PACAP also directly affects the pituitary gonadotrophs and modulates gonadotropin synthesis and release (Hart et al., 1992; Tsujii et al., 1994). In addition, it has been revealed that GnRH neurons or pituitary gonadotrophs possess PACAP receptors (Kanasaki et al., 2011; Olcese et al., 1997). We have previously reported that PACAP increased the expression of GnRH receptor, but not GnRH in GnRH-producing GT1-7 cell lines (Kanasaki et al., 2013). In the pituitary gonadotroph cell line LβT2, PACAP could stimulate gonadotropin subunit gene expression (Harada et al., 2007), and PACAP plays a role in GnRH pulse frequency-dependent gonadotropin subunit gene expression (Kanasaki et al., 2011).

It remains unknown whether PACAP could affect kisspeptin neurons in the hypothalamus. In rodents, kisspeptin neurons are located in two different regions of the hypothalamus, the arcuate nucleus (ARC) and the anteroventral periventricular (AVPV) region (Clarkson et al., 2009). Kisspeptin expression in the ARC region is inhibited by estradiol (E2), whereas that in the AVPV region is stimulated by E2 (Smith et al., 2005); therefore, kisspeptin neuronal populations in these different regions are thought to be a center of positive and negative feedback mechanisms by E2, respectively. Recently established hypothalamic cell models mHypoA-50 and mHypoA-55 contain kisspeptin neurons in the AVPV and ARC regions, respectively. An increase in Kiss-1 gene (which encodes kisspeptin) expression in mHypoA-50 cells and a reduction in Kiss-1 mRNA expression in mHypoA-55 cells by E2 have been observed under certain experimental conditions (Treen et al., 2016). Using these cell models, we examined the effect of PACAP on Kiss-1 gene expression.

2. Materials and methods

2.1. Materials

The following chemicals and reagents were obtained from the indicated sources: GIBCO fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA); PACAP38 and PACAP receptor antagonist, PACAP6-38 (Peptide Institute, Osaka, Japan); Dulbecco's modified Eagle's medium (DMEM), water-soluble E2, and penicillin-streptomycin (Sigma-Aldrich Co., St. Louis, MO); neurotensin (NT) and corticotropin-releasing hormone (CRH) (Life Technologies Japan., Ltd., Tokyo, Japan); and PKA inhibitor, H89 (Biomol, Plymouth, PA).

2.2. Cell culture and stimulation

Immortalized hypothalamic cell lines which were originated from adult mouse hypothalamic kisspeptin-containing neurons in the AVPV (mHypoA-50 cells) and ARC (mHypoA-55 cells) regions, were purchased from Cedarlane Laboratories under contract (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 with PACAP (10 nM or 100 nM), cells were cultured in high-glucose DMEM containing 1% heat-inactivated FBS and 1% penicillin-streptomycin with PACAP for 24 h. To examine the effect of E2, cells were cultured with E2 in phenol red–free DMEM supplemented with 1% charcoal-stripped FBS (Gemini Bio-Products, West Sacramento, CA). When examine the effects of PACAP6-38 and H89 on PACAP action, cells were preincubated in the presence of these reagents before PACAP stimulation. Then, mRNA was extracted and reverse transcribed.

2.3. RNA preparation, reverse transcription, and 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, PACAP, NT, and CRH mRNAs, after PCR amplification using primers for Kiss-1 (forward: 5′-ATGATCTCGCTGGCTTCTTGG-3′ and reverse: 5′-GGTTCACCACAGGTGCCATTTT-3′), PACAP (forward: 5′-GATGTCGCCCACGAAATCCT-3′ and reverse: 5′-GTATGCTATTCGGC GTCCTT-3′), NT (forward: 5′-GTGTGGACCTGCTTGTCAGA-3′ and reverse: 5′-TCATGCATGTCTCCTGCTTC-3′), and CRH (forward: 5′-ATCC GCATGGGTGAAGAATACT-3′ and reverse: 5′-TGGAAGGTGAGATCCA GAGAGA-3′), amplicons were electrophoresed in agarose gels and visualized with ethidium bromide staining. Kiss-1, PACAP, NT, and CRH mRNAs were measured using 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, PACAP, NT, and CRH, 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 (Wong and Medrano, 2005). 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 (Bustin et al., 2005). Briefly, for each sample, a ΔCt was calculated relative to the internal control using the following equation: ΔCt = ΔCt(gene) − Ct(GAPDH). To determine differences between experimental and control conditions, ΔΔCt was calculated as ΔCt(sample) − ΔCt(control). Relative mRNA levels were then calculated using the following equation: fold difference = $2^{\Delta\Delta Ct}$.

2.4. Western blotting analysis

Cell extracts were lysed on ice with RIPA buffer (phosphate-buffered saline, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) 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 (30 μg per well) was resolved in a 10% SDS polyacrylamide gel electrophoresis (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-PACAP antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) in Blotto overnight at 4 °C and washed three times for 10 min per wash with Tris-buffered saline/1% Tween. Subsequent incubation with horseradish peroxidase (HRP)-conjugated antibodies (1:15000 dilution) was performed for 1 h at room temperature in Blotto, with additional washes as appropriate. Following enhanced chemiluminescence detection (Amersham Biosciences), membranes were exposed to X-ray film (Fujifilm, Tokyo, Japan). Rat brain tissue was used as a positive control, and the

experimental protocol was approved by the animal care and use committee of the Experimental Animal Center for Integrated Research at Shimane University (IZ27-82).

2.5. Statistical analysis

All experiments were repeated independently at least three times. Experiments in all groups were performed in duplicate. Two samples were assayed in duplicate to determine mRNA expression. Six averages from three independent experiments were statistically analyzed. Data are expressed as the mean ± standard error of the mean. Statistical analysis was performed using one-way analysis of variance followed by Duncan's multiple range test, or Student's *t* test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of PACAP on Kiss-1 gene expression in hypothalamic cell models

To examine the direct effect of PACAP on Kiss-1 gene expression, two hypothalamic kisspeptin-expressing cell models were used. mHypoA-50 cells provide a model of kisspeptin-expressing cells in the AVPV region, whereas mHypoA-55 cells are used as a model of kisspeptin-expressing neurons in the ARC region (Treen et al., 2016). PACAP increased Kiss-1 gene expression in these cell models. Stimulation with 10 nM PACAP led to significant increases in Kiss-1 mRNA up to 2.69 ± 0.93-fold in mHypoA-50 cells and 4.89 ± 1.13-fold in mHypoA-55 cells (Fig. 1A and B). Another type of Kiss-1-producing cell line, rHypoE-8 cells which originate from fetal rat hypothalamus (Gingerich et al., 2009), also responded to PACAP by increasing Kiss-1 mRNA expression. Ten nM PACAP significantly increased Kiss-1 mRNA expression up to 3.25 ± 0.24-fold compared to non-stimulated cells (Fig. 1C).

3.2. Effect of the PACAP receptor antagonist PACAP6-38 on PACAP-induced Kiss-1 mRNA expression in both mHypoA-50 and mHypoA-55 cells

Next, we investigated the effect of PACAP6-38, a potent and competitive PAC1 receptor (PAC1R) and VPAC2 receptor (VPAC2R) antagonist, on PACAP-induced Kiss1 gene expression in these two cell models. Unexpectedly, PACAP6-38 itself increased the expression of Kiss-1 mRNA in both mHypoA-50 and mHypoA-55 cells. PACAP6-38 did not prevent the stimulatory effect of PACAP on Kiss-1 gene expression (Fig. 2A and B).

3.3. Effect of H89, a protein kinase A inhibitor, on PACAP-induced Kiss-1 gene expression

Kiss-1 gene expression induced by PACAP stimulation was completely inhibited in the presence of the protein kinase A (PKA) inhibitor H89. In the mHypoA-50 AVPV cell model, Kiss-1 gene expression was induced by PACAP, but its stimulatory effect was completely inhibited in the presence of H89. H89 alone decreased the expression of Kiss-1 gene compared to the non-stimulated control (Fig. 3A). Similarly, in mHypoA-55 ARC cells, PACAP-induced Kiss-1 gene expression was significantly inhibited in the presence of H89 (Fig. 3B).

3.4. Both mHypoA-50 and mHypoA-55 cells express PACAP

PACAP was expressed in these hypothalamic cell models. Using RT-PCR analysis with specific primers for PACAP, we demonstrated that PACAP gene was amplified in both mHypoA-50 and mHypoA-55 cells (Fig. 4A). Western blotting analysis using an antibody against PACAP also demonstrated an immunoreactive band in these cell models (Fig. 4B). Rat brain tissue was used as a positive control in this analysis.

3.5. Effect of E2 on PACAP mRNA expression in both mHypoA-50 and mHypoA-55 cells

It has been reported that mHypoA-50 and mHypoA-55 cells respond differently to E2 to regulate Kiss-1 gene expression (Treen et al., 2016). Next, we examined the effect of E2 on PACAP mRNA expression. In mHypoA-50 cells, 10 and 100 nM E2 significantly increased PACAP mRNA expression up to 2.78 ± 0.39-fold and 7.10 ± 2.87-fold, respectively (Fig. 5A). In contrast, PACAP gene expression in mHypoA-55 cells was not significantly increased by 10 and 100 nM E2 (Fig. 5B).

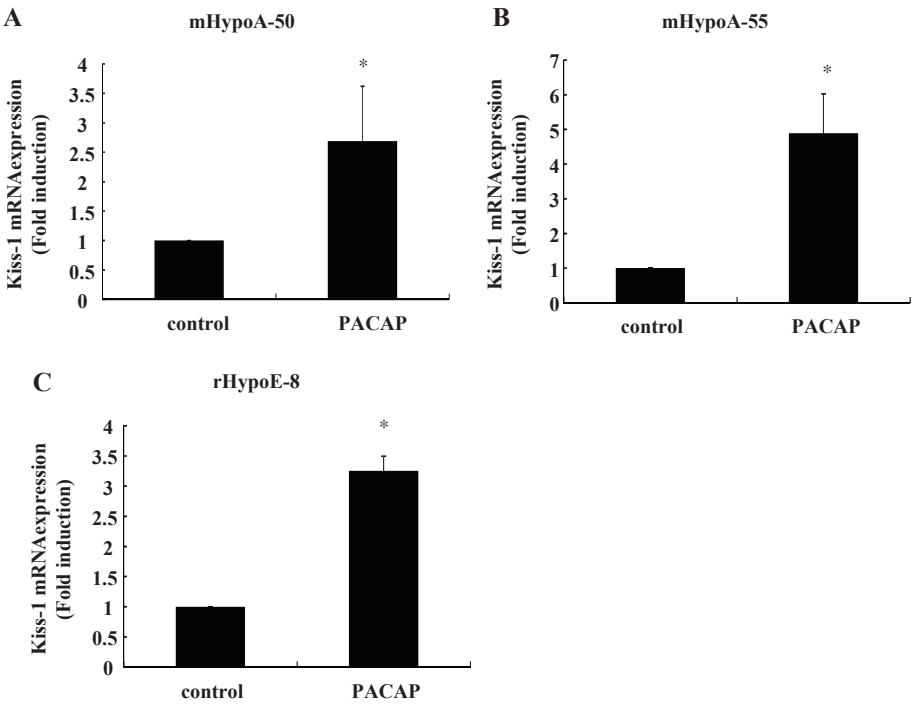

Fig. 1. Effect of PACAP on Kiss-1 gene expression in hypothalamic cell models. mHypoA-50 (A), mHypoA-55 (B), and rHypoE-8 cells (C) were stimulated with 10 nM PACAP for 24 h, after which mRNA was extracted and reverse transcribed. Kiss-1 mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold increase relative to unstimulated cells and presented as the mean ± standard error of the mean (SEM) of three independent experiments, each performed with duplicate samples. $^{*}P < 0.05$ vs. control.

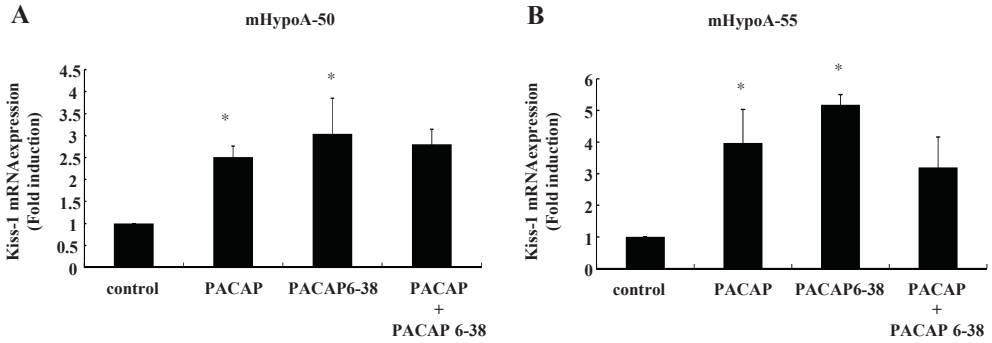

Fig. 2. Effect of PACAP receptor antagonist PACAP6-38 on PACAP-induced Kiss-1 mRNA expression in mHypoA-50 and mHypoA-55 cells. mHypoA-50 (A) and mHypoA-55 (B) were stimulated with PACAP (10 nM), PACAP6-38 (100 nM), or both for 24 h. For co-stimulation with PACAP and PACAP6-38, cells were pre-incubated in the presence of PACAP6-38 for 1 h. Then, mRNA was extracted and reverse transcribed. Kiss-1 mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold increase relative to unstimulated cells and presented as the mean ± SEM of three independent experiments, each performed with duplicate samples. $^{*}P < 0.05$ vs. control.

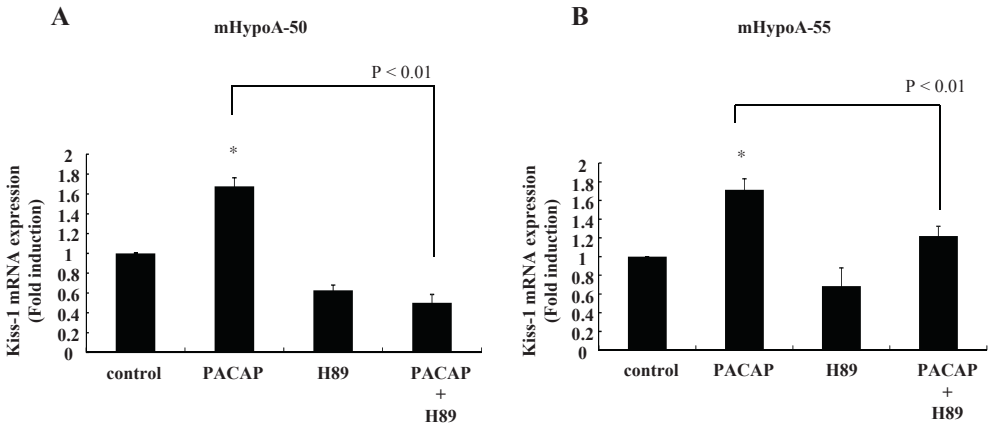

Fig. 3. Effect of H89, a krptein kinase A inhibitor, on PACAP-induced Kiss-1 gene expression. mHypoA-50 (A) and mHypoA-55 (B) were stimulated with PACAP in the presence or absence of 100 nM H89 for 24 h. Cells were preincubated in the presence of H89 for 1 h before stimulation. Then, mRNA was extracted and reverse transcribed. Kiss-1 mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold increase relative to unstimulated cells and presented as the mean ± SEM of three independent experiments, each performed with duplicate samples. $^{*}P < 0.05$ vs. control. The differences between PACAP and PACAP + H89 were statistically significant in both cell models ($P < 0.01$).

3.6. Effect of PACAP on the expression of CRH and NT

Several lines of evidence suggest that both CRH and NT have an effect on the HPG axis. PACAP can stimulate the expression of both CRH and NT in mHypoA-50 and mHypoA-55 cells. CRH mRNA expression was significantly increased by PACAP stimulation at 100 nM up to 7.23 ± 2.17-fold in the mHypoA-50 AVPV model. A non-significant increase in CRH mRNA was induced by 10 nM PACAP (3.25 ± 0.65-fold). PACAP also increased CRH expression in the mHypoA-55 ARC model; CRH mRNA expression was significantly increased up to 5.71 ± 0.81-fold and 3.12 ± 0.73-fold by 10 and 100 nM PACAP, respectively (Fig. 6A and B). Similarly, NT was also upregulated by PACAP stimulation in these cells. NT mRNA expression was significantly increased up to 4.8 ± 0.84-fold by 100 nM PACAP in mHypoA-50 cells, and 5.64 ± 2.04-fold by 10 nM PACAP in mHypoA-55 cells (Fig. 7A and B).

4. Discussion

Nearly 30 years ago, Arimura and colleagues isolated a novel neuropeptide from sheep hypothalamic extracts (Miyata et al., 1989). This neuropeptide, named PACAP, has a variety of functions, but it is largely unknown how this peptide influences the hypothalamic factors that control the HPG axis. In the present study, we used two different hypothalamic cell lines, mHypoA-50 and mHypoA-55, as neuronal models of AVPV and ARC regions of the murine hypothalamus. We found that 1) PACAP could increase the expression of Kiss-1 gene, whose product has been shown to control GnRH synthesis and release; 2) PACAP receptor antagonist, PACAP6-38 did not antagonize the effect of PACAP, but exerted an agonistic effect on Kiss-1 gene expression in these cell models; 3) PACAP was expressed in these hypothalamic cell models; 4) Expression of PACAP in these cells was upregulated by E2; and 5) PACAP was able to stimulate CRH and NT gene expressions.

Because the origins of mHypoA-50 and mHypo-A55 cell lines are distinct, these two kisspeptin-expressing neuronal cells have different characteristics although both express kisspeptin. For example, substance P, neurokinin B, and dynorphin, all of which are expressed in ARC nucleus, were expressed in the mHypoA-55 cell line. By contrast, mHypoA-50 neurons express tyrosine hydroxylase, but not neurokinin B and dynorphin, supporting their putative role as AVPV kisspeptin neurons (Treen et al., 2016). In addition, we found that several genes of neuropeptides such as PACAP, CRH, and NT were also expressed and regulated within these cells. These observations suggest that these neuropeptides expressed in these cells may be active in the AVPV or ARC regions of the hypothalamus.

Although PACAP has variety of functions in the CNS (Vaudry et al., 2009), little is known about the function of this peptide in the HPG axis. Previous studies showed that PACAP may modulate the HPG axis by acting on GnRH neurons, but the reported results were contradictory. Intracerebroventricular injection of PACAP or injection into the medial basal hypothalamus which houses the GnRH neurons suppressed GnRH pulsatility in ovariectomized ewes (Anderson et al., 1996). Similarly, it has been reported that intracerebroventricular administration of PACAP inhibited the LH surge and ovulation mediated through CRH and endogenous opioids (Koves et al., 2003). Furthermore, PACAP administration to neonatal female rats delayed puberty (Koves et al., 2003). These observations suggested that PACAP has an inhibitory effect on the regulators of the HPG axis. On the other hand, it has been reported that intravenous administration of PACAP to male rats slightly increased GnRH mRNA level in the hypothalamus (Li et al., 1996) and that intra-arterial and intracerebroventricular injection of PACAP stimulated LH release in intact adult rats (Osuga et al., 1992). It is generally agreed that hypothalamic kisspeptin neurons are located in two different regions of the hypothalamus and control the activity of GnRH neurons. Because PACAP stimulated an increase in Kiss-1 gene expression in two hypothalamic cell models, it is conceivable that PACAP could maintain the function of kisspeptin-expressing neurons by stimulating Kiss-1 gene expression. PACAP-increased Kiss-1 expression

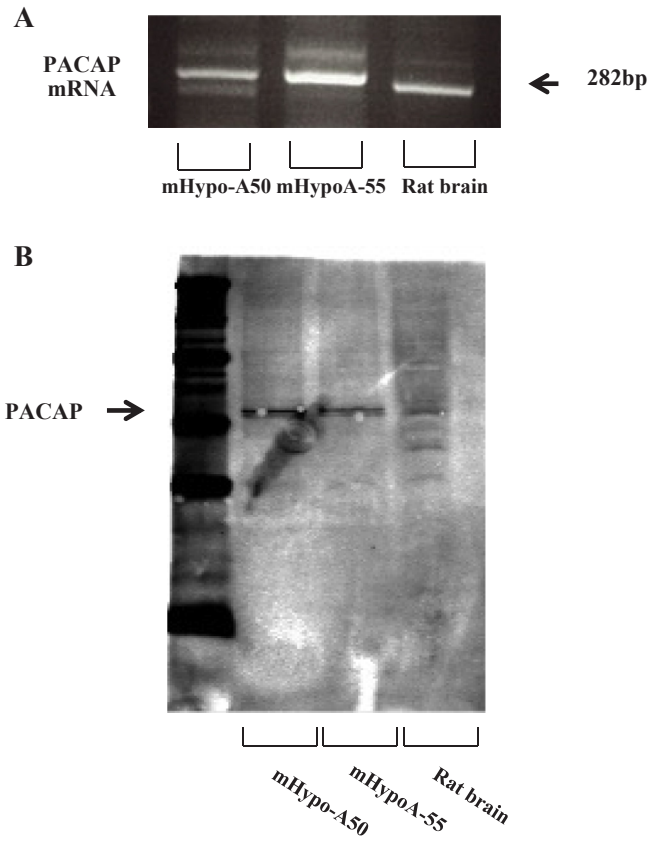

Fig. 4. Expression of PACAP in mHypoA-50 and mHypoA-55 cells. (A) Total RNA was prepared and RT-PCR was performed for 40 cycles using PACAP-specific primers. PCR products were resolved in a 3.0% agarose gel and visualized with ethidium bromide staining. (B) Cell lysates (30 μg) from mHypoA-50 cells and mHypoA-55 cells were analyzed by SDS-PAGE followed by immunoblotting and incubation with antibodies against PACAP. The bands were visualized using HRP-conjugated secondary antibody.

was not prevented in either cell model in the presence of PACAP6-38, a potent antagonist of the PACAP receptor, PAC1R and VPAC2R (Castorina et al., 2014; Robberecht et al., 1992). PACAP6-38 did not act as a PACAP receptor antagonist, but it exerted a similar effect to PACAP on Kiss-1 expression. Although PACAP6-38 acts as an antagonist of PACAP, there is also evidence that in certain models PACAP6-38 can behave similarly to PACAP (Ichinose et al., 1995; Juhasz et al., 2014; Reglodi et al., 2008; Wojcieszak and Zawilska, 2014). In hypothalamic kisspeptin neurons, both PACAP and PACAP6-38 similarly increased Kiss-1 gene expression.

From the observations that both mHypoA-50 and mHypoA-55 cells express PACAP and that PACAP could modulate Kiss-1 gene expression, it is plausible that PACAP within these cells affects Kiss-1 expression in an autocrine or paracrine fashion. Unfortunately, at present, we are not able to determine whether PACAP and Kiss-1 are co-expressed in the same cells or are expressed in different cells among these cells. Because these cells originate from hypothalamic neurons located in the AVPV and ARC regions, it might contain several different neuronal cells. Furthermore, although we measured CRH and NT expression by PACAP stimulation in these cells, it still unknown whether these neuropeptides are co-expressed in single cells. However, we were able to investigate the direct effect of PACAP on the expression of these neuropeptides. NT was first isolated from bovine hypothalamus based on its potent vascular effects (Carraway and Leeman, 1973), and NT is abundantly expressed in the CNS with particularly high expression in the hypothalamus, including in the AVPV and ARC regions (Carraway and Leeman, 1976; Rostene and Alexander, 1997). On the other hand, CRH is an important physiological adrenocorticotropic hormone-releasing factor that is expressed in hypothalamic nuclei. Both NT and CRH have been found to have an influence on the function of the HPG axis. For example, E2 induces the expression of NT gene in AVPV lesions in rats (Alexander et al., 1989; Smith and Wise, 2001). Administration of NT evokes an LH surge, and antiserum against NT blocks this LH surge (Akema et al., 1987; Ferris et al., 1984). CRH expression in the hypothalamus has been reported to be increased after administration of E2 in ovariectomized monkeys (Kerdelhue et al., 1995). Furthermore, several studies implicated CRH as a prime candidate for stress-induced suppression of pulsatile release of GnRH (Cates et al., 2004; Li et al., 2006). Although PACAP has a direct effect on Kiss-1 expression, PACAP could influence the HPG axis indirectly by changing NT or CRH expression within the hypothalamus. Indeed, PACAP expression was significantly upregulated by E2 in mHypoA-50 cells, but not in mHypoA-55 cells, suggesting that PACAP plays a role in E2-induced positive feedback regulation in the AVPV region of the hypothalamus because mHypoA-50 cells are an AVPV neuronal model.

As described above, in rodents, Kiss-1 expression in the AVPV region of the hypothalamus is stimulated by E2, whereas Kiss-1 expression in the ARC region is inhibited by E2 (Smith et al., 2005). A similar phenomenon was also observed in mHypoA-50 AVPV and mHypoA-55 ARC cell models under certain experimental condition (Treen et al., 2016). We have recently found that stimulation of mHypoA-50 cells with NT increased Kiss-1 expression; by contrast, mHypoA-55 cells were inhibited by NT stimulation. Similarly, we observed that CRH stimulated Kiss-1 expression in mHypoA-50 cells, but reduced Kiss-1 in mHypoA-55 cells. Furthermore, we observed that both NT and CRH were upregulated in the presence of E2 in both cell lines (data not shown). Because NT and CRH have opposite effects on Kiss-1 gene expression under the influence of E2 in mHypoA-50 and mHypoA-55 cells, we speculate that both NT and CRH had a role in E2-induced positive and negative feedback mechanisms.

In this study, we found that PACAP increased hypothalamic Kiss-1 expression in two different cell models from the AVPV and ARC regions

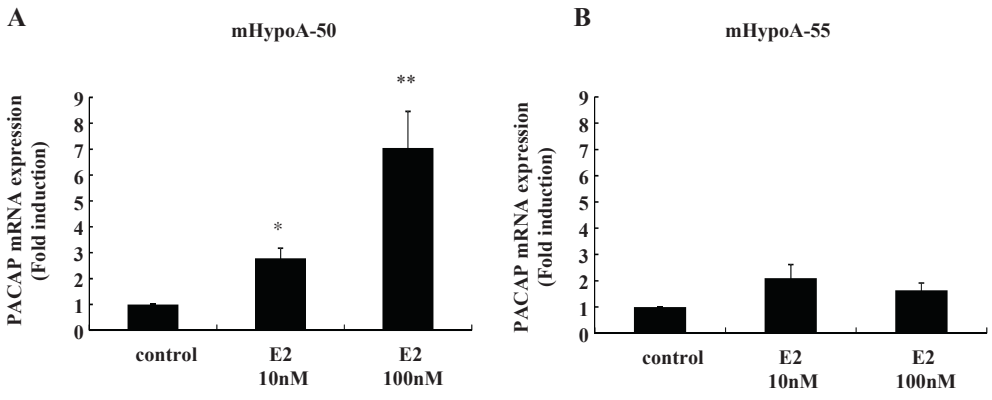

Fig. 5. Effect of E2 on PACAP mRNA expression in mHypoA-50 and mHypoA-55 cells. mHypoA-50 cells (A) and mHypoA-55 cells (B) were stimulated with 10 nM and 100 nM E2 for 24 h, after which mRNA was extracted and reverse transcribed. PACAP mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold increase relative to unstimulated cells and presented as the mean ± SEM of three independent experiments, each performed with duplicate samples. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control.

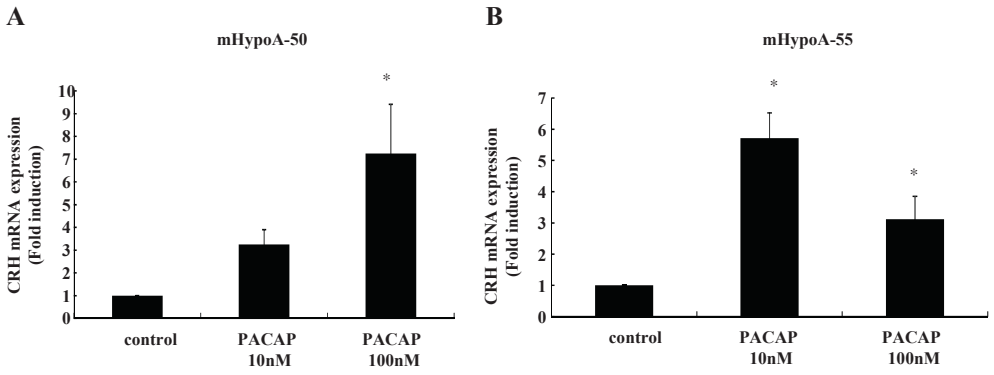

Fig. 6. Effect of PACAP on CRH mRNA expression in mHypoA-50 and mHypoA-55 cells. mHypoA-50 cells (A) and mHypoA-55 cells (B) were stimulated with 10 nM and 100 nM PACAP for 24 h, after which mRNA was extracted and reverse transcribed. CRH mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold increase relative to unstimulated cells and presented as the mean ± SEM of three independent experiments, each performed with duplicate samples. $^{*}P < 0.05$ vs. control.

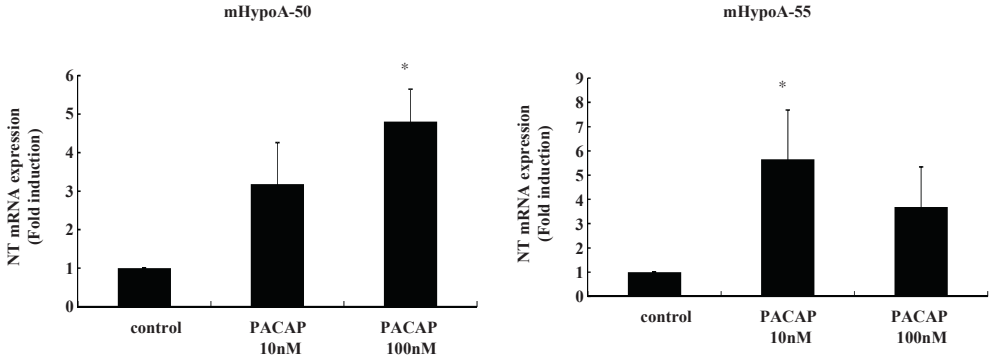

Fig. 7. Effect of PACAP on NT mRNA expression in mHypoA-50 and mHypoA-55 cells. mHypoA-50 cells (A) and mHypoA-55 cells (B) were stimulated with 10 nM and 100 nM PACAP for 24 h, after which mRNA was extracted and reverse transcribed. NT mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold increase relative to unstimulated cells and presented as the mean ± SEM of three independent experiments, each performed with duplicate samples. $^{*}P < 0.05$ vs. control.

of the hypothalamus. PACAP was expressed in these cells and was up-regulated by E2 in the AVPV, but not the ARC, cell model. Because PACAP could increase expression of NT and CRH, which differentially affect Kiss-1 expression in these two hypothalamic cell models, PACAP might have a role in the feedback mechanisms of E2.

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 (to H. K. and A.O.)

Conflict of interest

The authors have nothing to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.10.006.

References

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

Alexander, M.J., Dobner, P.R., Miller, M.A., Bullock, B.P., Dorsa, D.M., Leeman, S.E., 1989. Estrogen induces neurotensin/neuromedin N messenger ribonucleic acid in a preoptic nucleus essential for the preovulatory surge of luteinizing hormone in the rat. Endocrinology 125, 2111–2117.

Anderson, S.T., Sawangjaroen, K., Curlewis, J.D., 1996. Pituitary adenylate cyclase-activating polypeptide acts within the medial basal hypothalamus to inhibit prolactin and luteinizing hormone secretion. Endocrinology 137, 3424–3429.

Bustin, S.A., Benes, V., Nolan, T., Pfaffl, M.W., 2005. Quantitative real-time RT-PCR–a perspective. J. Mole. Endocrinol. 34, 597–601.

Carraway, R., Leeman, S.E., 1973. The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami. J. Biol. Chem. 248, 6854–6861.

Carraway, R., Leeman, S.E., 1976. Characterization of radioimmunoassayable neurotensin in the rat. Its differential distribution in the central nervous system, small intestine, and stomach. J. Biol. Chem. 251, 7045–7052.

Castorina, A., Scuderi, S., D'Amico, A.G., Drago, F., D'Agata, V., 2014. PACAP and VIP increase the expression of myelin-related proteins in rat schwannoma cells: involvement of PAC1/VPAC2 receptor-mediated activation of PI3K/Akt signaling pathways. Exp. Cell Res. 322, 108–121.

Cates, P.S., Li, X.F., O'Byrne, K.T., 2004. 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 7, 113–118.

Choi, E.J., Ha, C.M., Kim, M.S., Kang, J.H., Park, S.K., Choi, W.S., Kang, S.G., Lee, B.J., 2000. Central administration of an antisense oligodeoxynucleotide against type I pituitary adenylate cyclase-activating polypeptide receptor suppresses synthetic activities of LHRH-LH axis during the pubertal process. Brain Res. Mol. Brain Res. 80, 35–45.

Clarkson, J., d'Anglemont de Tassigny, X., Colledge, W.H., Caraty, A., Herbison, A.E., 2009. Distribution of kisspeptin neurones in the adult female mouse brain. J. Neuroendocrinol. 21, 673–682.

Crowley Jr., W.F., Filicori, M., Spratt, D.I., Santoro, N.F., 1985. The physiology of gonadotropin-releasing hormone (GnRH) secretion in men and women. Recent Prog. Horm. Res. 41, 473–531.

de Roux, N., Genin, E., Carel, J.C., Matsuda, F., Chaussain, J.L., Milgrom, E., 2003. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc. Natl. Acad. Sci. USA 100, 10972–10976.

Ferris, C.F., Pan, J.X., Singer, E.A., Boyd, N.D., Carraway, R.E., Leeman, S.E., 1984. Stimulation of luteinizing hormone release after stereotaxic microinjection of neurotensin into the medial preoptic area of rats. Neuroendocrinology 38, 145–151.

Gharib, S.D., Wierman, M.E., Shupnik, M.A., Chin, W.W., 1990. Molecular biology of the pituitary gonadotropins. Endocr. Rev. 11, 177–199.

Gingerich, S., Wang, X., Lee, P.K., Dhillon, S.S., Chalmers, J.A., Koletar, M.M., Belsham, D.D., 2009. The generation of an array of clonal, immortalized cell models from the rat hypothalamus: analysis of melatonin effects on kisspeptin and gonadotropin-inhibitory hormone neurons. Neuroscience 162, 1134–1140.

Gonzalez, B.J., Basille, M., Vaudry, D., Fournier, A., Vaudry, H., 1998. Pituitary adenylate cyclase-activating polypeptide. Ann. Endocrinol. (Paris) 59, 364–405.

Harada, T., Kanasaki, H., Mutiara, S., Oride, A., Miyazaki, K., 2007. Cyclic adenosine 3',5'monophosphate/protein kinase A and mitogen-activated protein kinase 3/1 pathways are involved in adenylate cyclase-activating polypeptide 1-induced common alpha-glycoprotein subunit gene (Cga) expression in mouse pituitary gonadotroph LbetaT2 cells. Biol. Reprod. 77, 707–716.

Hart, G.R., Gowing, H., Burrin, J.M., 1992. Effects of a novel hypothalamic peptide, pituitary adenylate cyclase-activating polypeptide, on pituitary hormone release in rats. J. Endocrinol. 134, 33–41.

Ichinose, M., Asai, M., Imai, K., Sawada, M., 1995. Enhancement of phagocytosis in mouse macrophages by pituitary adenylate cyclase activating polypeptide (PACAP) and related peptides. Immunopharmacology 30, 217–224.

Juhasz, T., Matta, C., Katona, E., Somogyi, C., Takacs, R., Gergely, P., Csernoch, L., Panyi, G., Toth, G., Reglodi, D., Tamas, A., Zakany, R., 2014. Pituitary adenylate cyclase activating polypeptide (PACAP) signalling exerts chondrogenesis promoting and

protecting effects: implication of calcineurin as a downstream target. PLoS One 9, e91541.
Kanasaki, H., Mijiddorj, T., Sukhbaatar, U., Oride, A., Miyazaki, K., 2013. Pituitary adenylate cyclase-activating polypeptide (PACAP) increases expression of the gonadotropin-releasing hormone (GnRH) receptor in GnRH-producing GT1-7 cells overexpressing PACAP type I receptor. Gen. Comp. Endocrinol. 193, 95–102.
Kanasaki, H., Purwana, I.N., Mijiddorj, T., Oride, A., Miyazaki, K., 2011. Possible involvement of PACAP and PACAP type 1 receptor in GnRH-induced FSH beta-subunit gene expression. Regul Pept. 167, 227–232.
Kerdelhue, B., Jones, G.S., Gordon, K., Seltman, H., Lenoir, V., Melik Parsadaniantz, S., Williams, R.F., Hodgen, G.D., 1995. 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. 42, 228–235.
Koves, K., Kantor, O., Molnar, J., Heinzlmann, A., Szabo, E., Szabo, F., Nemeskeri, A., Horvath, J., Arimura, A., 2003. The role of PACAP in gonadotropic hormone secretion at hypothalamic and pituitary levels. J. Mol. Neurosci. 20, 141–152.
Li, S., Grinevich, V., Fournier, A., Pelletier, G., 1996. Effects of pituitary adenylate cyclase-activating polypeptide (PACAP) on gonadotropin-releasing hormone and somatostatin gene expression in the rat brain. Brain Res. Mol. Brain Res. 41, 157–162.
Li, X.F., Bowe, J.E., Kinsey-Jones, J.S., Brain, S.D., Lightman, S.L., O'Byrne, K.T., 2006. 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. 18, 602–610.
Maggi, R., Cariboni, A.M., Marelli, M.M., Moretti, R.M., Andre, V., Marzagalli, M., Limonta, P., 2016. GnRH and GnRH receptors in the pathophysiology of the human female reproductive system. Hum. Reprod. Update 22.
Miyata, A., Arimura, A., Dahl, R.R., Minamino, N., Uehara, A., Jiang, L., Culler, M.D., Coy, D.H., 1989. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochem. Biophys. Res. Commun. 164, 567–574.
Okada, R., Yamamoto, K., Ito, Y., Mochida, H., Tonon, M.C., Fournier, A., Leprince, J., Vaudry, H., Kikuyama, S., 2007. VIP and PACAP stimulate TSH release from the bullfrog pituitary. Peptides 28, 1784–1789.
Olcese, J., McArdle, C.A., Middendorff, R., Greenland, K., 1997. Pituitary adenylate cyclase-activating peptide and vasoactive intestinal peptide receptor expression in immortalized LHRH neurons. J. Neuroendocrinol. 9, 937–943.
Osuga, Y., Mitsuhashi, N., Mizuno, M., 1992. In vivo effect of pituitary adenylate cyclase activating polypeptide 38 (PACAP 38) on the secretion of luteinizing hormone (LH) in male rats. Endocrinol. Jpn. 39, 153–156.
Reglodi, D., Borzsei, R., Bagoly, T., Boronkai, A., Racz, B., Tamas, A., Kiss, P., Horvath, G., Brubel, R., Nemeth, J., Toth, G., Helyes, Z., 2008. Agonistic behavior of PACAP6-38 on sensory nerve terminals and cytotrophoblast cells. J. Mol. Neurosci. 36, 270–278.
Robberecht, P., Gourlet, P., De Neef, P., Woussen-Colle, M.C., Vandermeers-Piret, M.C., Vandermeers, A., Christophe, J., 1992. Structural requirements for the occupancy of pituitary adenylate-cyclase-activating-peptide (PACAP) receptors and adenylate cyclase activation in human neuroblastoma NB-OK-1 cell membranes. Discovery of PACAP(6–38) as a potent antagonist. Eur. J. Biochem. 207, 239–246.
Rostene, W.H., Alexander, M.J., 1997. Neurotensin and neuroendocrine regulation. Front. Neuroendocrinol. 18, 115–173.
Seminara, S.B., Messager, S., Chatzidaki, E.E., Thresher, R.R., Acierno Jr., J.S., Shagoury, J.K., Bo-Abbas, Y., Kuohung, W., Schwinof, K.M., Hendrick, A.G., Zahn, D., Dixon, J., Kaiser, U.B., Slaugenhaupt, S.A., Gusella, J.F., O'Rahilly, S., Carlton, M.B., Crowley Jr., W.F., Aparicio, S.A., Colledge, W.H., 2003. The GPR54 gene as a regulator of puberty. N Engl. J. Med. 349, 1614–1627.
Smith, J.T., Cunningham, M.J., Rissman, E.F., Clifton, D.K., Steiner, R.A., 2005. Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 146, 3686–3692.
Smith, M.J., Wise, P.M., 2001. Neurotensin gene expression increases during proestrus in the rostral medial preoptic nucleus: potential for direct communication with gonadotropin-releasing hormone neurons. Endocrinology 142, 3006–3013.
Szabo, F., Horvath, J., Heinzlmann, A., Arimura, A., Koves, K., 2002. Neonatal PACAP administration in rats delays puberty through the influence of the LHRH neuronal system. Regul. Pept. 109, 49–55.
Treen, A.K., Luo, V., Chalmers, J.A., Dalvi, P.S., Tran, D., Ye, W., Kim, G.L., Friedman, Z., Belsham, D.D., 2016. Divergent regulation of ER and kiss genes by 17beta-estradiol in hypothalamic ARC versus AVPV models. Mol. Endocrinol. 30, 217–233.
Tsujii, T., Ishizaka, K., Winters, S.J., 1994. Effects of pituitary adenylate cyclase-activating polypeptide on gonadotropin secretion and subunit messenger ribonucleic acids in perifused rat pituitary cells. Endocrinology 135, 826–833.
Vaudry, D., Falluel-Morel, A., Bourgault, S., Basille, M., Burel, D., Wurtz, O., Fournier, A., Chow, B.K., Hashimoto, H., Galas, L., Vaudry, H., 2009. Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. Pharmacol. Rev. 61, 283–357.
Wojcieszak, J., Zawilska, J.B., 2014. PACAP38 and PACAP6-38 exert cytotoxic activity against human retinoblastoma Y79 cells. J. Mol. Neurosci. 54, 463–468.
Wong, M.L., Medrano, J.F., 2005. Real-time PCR for mRNA quantitation. BioTechniques 39, 75–85.