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Roles of intracerebral activin, inhibin, and follistatin in the regulation of Kiss-1 gene expression: Studies using primary cultures of fetal rat neuronal cells☆

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A B S T R A C T

Hypothalamic kisspeptin, encoded by the Kiss-1 gene, governs the hypothalamic-pituitary-gonadal axis by directly regulating the release of gonadotropin-releasing hormone. In this study, we examined the roles of activin, inhibin, and follistatin in the regulation of Kiss-1 gene expression using primary cultures of fetal rat neuronal cells, which express the Kiss-1 gene and kisspeptin. Stimulation with activin significantly increased Kiss-1 gene expression in these cultures by 2.02 ± 0.39-fold. In contrast, a significant decrease in Kiss-1 gene expression was observed with inhibin A and follistatin treatment. Inhibin B did not modulate Kiss-1 gene expression. Activin, inhibin, and follistatin were also expressed in fetal rat brain cultures and their expression was controlled by estradiol (E2). The inhibin α, βA, and βB subunits were upregulated by E2. Similarly, follistatin gene expression was significantly increased by E2 in these cells. Our results suggest the possibility that activin, inhibin, and follistatin expressed in the brain participate in the E2-induced feedback control of the hypothalamic-pituitary-gonadal axis.

1. Introduction

Hypothalamic kisspeptin-expressing neurons (Kiss-1 neurons) are positioned at the highest level of the hypothalamic-pituitary-gonadal (HPG) axis and govern reproductive function primarily by controlling the release of gonadotropin-releasing hormone (GnRH) from GnRH-expressing neurons in the preoptic area of the hypothalamus [1]. Kiss-1 neurons are located in two different areas of the hypothalamus, namely, the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV), and project to GnRH-expressing neurons [2]. Accumulating evidence indicates that Kiss-1 neurons in the ARC coordinate the pulsatile secretion of GnRH, which ultimately maintains the pulsatile secretion of gonadotropins from the anterior pituitary. In contrast, Kiss-1 neurons in the AVPV evoke a surge of GnRH/luteinizing hormone to induce ovulation [1]. Because Kiss-1 expression is upregulated and downregulated by estradiol (E2) in the AVPV and ARC, respectively [3, 4], Kiss-1 neurons in these regions are suggested to be at the center of

E2-induced positive and negative feedback mechanisms.

Activin, inhibin, and follistatin also participate in the control of the HPG axis. Activin was originally isolated from ovarian follicular fluid and is believed to act as an autocrine/paracrine mediator within the ovary in the regulation of follicular maturation and steroid synthesis [5, 6]. Activin also has a specific stimulatory effect on follicle-stimulating hormone (FSH) release from the pituitary gland [7]. In contrast, inhibin and follistatin, both of which were also identified from follicular fluid, have an antagonistic effect on activin, and inhibit its effect within the ovary and pituitary gland [6,8,9].

Although activin, inhibin, and follistatin are produced predominantly in the ovary, they are also expressed in the central nervous system, including the hypothalamus [10]. Activin consists of β-subunit heterodimers that are encoded by the *Inhba* and *Inhbb* genes, producing activin A (βA/βA), B (βB/βB), and AB (βA/βB) [11]. Inhibin is a dimeric protein consisting of an α protein subunit and one of two β subunits, βA or βB, thus giving rise to inhibin A (α/βA) or B (α/βB) [12]. The presence

of activin/inhibin subunits or follistatin in the brain suggests these proteins play some physiological roles within the brain.

We have reported that inhibin subunits and follistatin are expressed in a Kiss-1-expressing neuronal cell model, mHypoA-55 cells, which originate from the ARC region of the hypothalamus. Using these cells, we showed that the gene expression of the inhibin α subunit and follistatin was upregulated by E2. In addition, we revealed that Kiss-1 gene expression in mHypoA-55 cells was increased by exogenous activin stimulation, while it was repressed by exogenous inhibin A or follistatin stimulation [13]. However, it remains unclear whether Kiss-1 gene expression is regulated by activin, inhibin, and follistatin and whether their expression is influenced by circulating E2 *in vivo*.

In this study, we used primary cultures of neuronal cells obtained from whole fetal rat brain. These primary cultures contained a variety of neuronal types, including those expressing Kiss-1, activin, inhibin, and follistatin. Using this cell model, we ascertained that Kiss-1 gene expression is regulated by activin, inhibin, and follistatin and that the genes for activin/inhibin and follistatin are regulated by E2.

2. Materials and methods

2.1. Materials

The following chemicals and reagents were obtained from the indicated sources: Dulbecco's modified Eagle's medium (DMEM), follistatin, water-soluble E2, and penicillin-streptomycin (Sigma-Aldrich Co., St. Louis, MO); activin A and inhibin B (Abcam, Cambridge, MA); inhibin A (R&D Systems, Inc., Minneapolis, MN); and fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA).

2.2. Primary culture of neuronal cells from fetal rat brain

Six to eight fetal rat brains were obtained from fetuses from a female rat at 16–18 days of gestation under deep sodium pentobarbital anesthesia. Whole brains from fetal rats were excised and minced before incubation in CMF-HBSS containing 10 mg/mL trypsin and 2 mg/mL collagenase (Nitta Gelatin, Osaka, Japan) for 15 min at 37 °C. The samples were then incubated in an identical solution containing 0.5 μg/mL DNase I (Boehringer-Mannheim, Mannheim, Germany) for 5 min at 37 °C. After incubation in CMF-HBSS containing 5 mM EDTA (Wako Pure Chemicals, Osaka, Japan) for 5 min 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, NJ), and collected by centrifugation. The pellet was resuspended and 2.0–3.0×10^5 cells were cultured on a 35-mm culture dish in DMEM with 10% FBS and 1% penicillin-streptomycin until use. This protocol was approved by the ethics committee of the Experimental Animal Center for Integrated Research at Shimane University (IZ27-82).

2.3. Cell culture and stimulation

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, the cells were used for each experiment. When stimulated with the test reagents, the cells were incubated with or without (control) test reagents in high-glucose DMEM containing 1% heat-inactivated FBS and 1% penicillin-streptomycin at the indicated concentrations. When stimulated with E2, the cells were cultured with E2 in phenol red-free DMEM supplemented with 1% charcoal-stripped FBS (Gemini Bio-Products, West Sacramento, CA).

2.4. 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 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 inhibin α, βA, and βB subunits and follistatin mRNAs, after PCR amplification using primers for inhibin α (forward: 5′-GTGGGGAGGTCCTA-GACAGA-3′ and reverse: 5′-GTGGGGATGGCCGGAATACA-3′), inhibin βA (forward: 5′-GGAGTGGATGGCAAGGTCAACA-3′ and reverse: 5′-GTGGGCACACAGCATGACTTA-3′), inhibin βB (forward: 5′-GGTCCGCCTGTACTTCTTCGTCT-3′ and reverse: 5′-GGTATGC-CAGCCGCTACGTT-3′), and follistatin (5′-GTGACAATGCCACATACGCC-3′ and reverse: 5′-GCCTCTGCAGTTACGCAATAA-3′), amplicons were electrophoresed in agarose gels and visualized with ethidium bromide staining. cDNAs from rat ovary and/or rat anterior pituitary gland were used as positive controls. Quantification of Kiss-1, inhibin α, βA, and βB subunits, and follistatin mRNAs were obtained through quantitative real-time PCR (ABI Prism 7000; PerkinElmer 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 mouse Kiss-1 (forward: 5′-ATGATCTCGCTGGCTTCTTGG-3′; reverse: 5′-GGTTCACCACAGGTGCCATTTT-3′), inhibin α, βA, and βB subunits, and follistatin, 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°C-95 °C). To determine PCR efficiency, a 10-fold serial dilution of cDNA was performed as previously described [14]. 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 calculated using the comparative threshold cycle (Ct) method [15]. Briefly, for each sample, a ΔCt value was calculated to normalize to the internal control using the following equation: ΔCt = ΔCt (gene) – Ct (GAPDH). To identify differences between the experimental and control conditions, ΔΔCt was calculated as ΔCt (sample) – ΔCt (control). Relative mRNA levels were calculated using the following equation: fold difference = $2^{\Delta\Delta Ct}$.

2.5. Western blot analysis

Primary cultures of fetal rat brain were lysed on ice with RIPA buffer (phosphate-buffered saline, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) containing 0.1 mg/mL phenylmethyl sulfonyl fluoride, 30 mg/mL aprotinin, and 1 mM sodium orthovanadate, scraped for 20 s, and centrifuged at $14,000 \times g$ 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 by 10% SDS-polyacrylamide gel electrophoresis (PAGE) 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). The membranes were incubated with an anti-kisspeptin antibody (1:100 dilution; Abcam), anti-inhibin α antibody (1:100 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX), anti-inhibin βA antibody (1:100 dilution; Santa Cruz Biotechnology,

Inc.), anti-inhibin βB antibody (1:1000 dilution; Abcam), or anti-follistatin antibody (1:100 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 20. Subsequent incubation with horseradish peroxidase-conjugated antibodies was performed for 1 h at room temperature in Blotto, and additional washes were performed as needed. Following enhanced chemiluminescence detection (Amersham Biosciences), the membranes were exposed to X-ray film (Fujifilm, Tokyo, Japan). Tissues from rat ovary and/or rat anterior pituitary gland were used as positive controls.
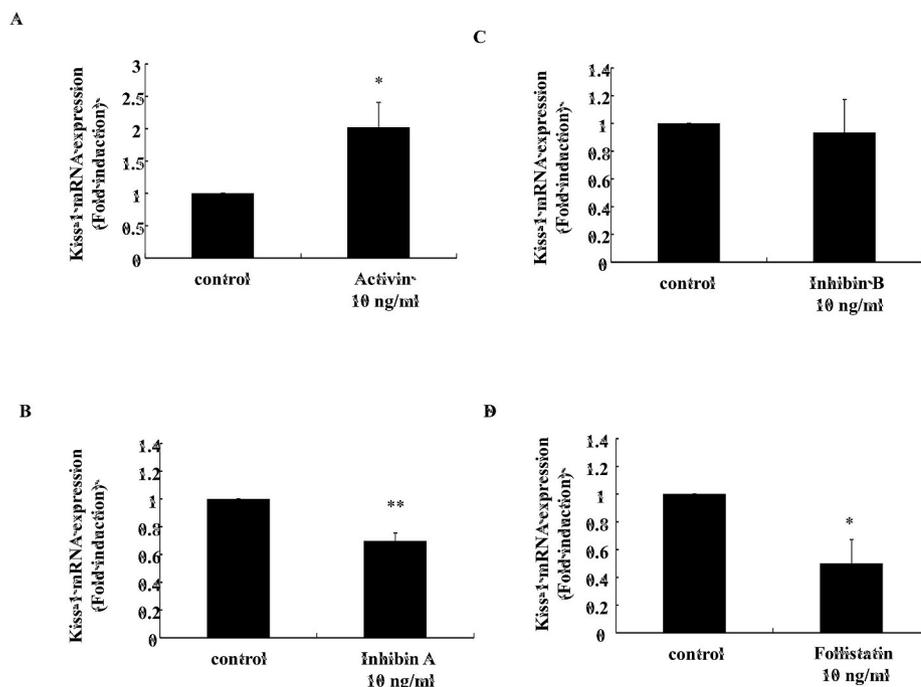
2.6. Statistical analysis

All experiments were repeated independently at least three times. Each experiment in each experimental group was performed using duplicate samples. When we determined mRNA expression, two samples were assayed in duplicate. Six averages from three independent experiments were statistically analyzed. Data are expressed as mean ± standard error of the 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.

3. Results

3.1. Effect of exogenous activin, inhibin and follistatin stimulation on Kiss-1 gene expression in primary cultures of fetal rat brain

In neuronal cell cultures obtained from whole fetal rat brains, Kiss-1-expressing neurons were detected; these cells also contained GnRH-expressing neurons [16]. Exogenous activin stimulation increased Kiss-1 gene expression in these primary cultures by 2.02 ± 0.39-fold compared to non-stimulated cells (Fig. 1A). In contrast, inhibin A stimulation inhibited Kiss-1 gene expression by 0.70 ± 0.06-fold (Fig. 1B); however, inhibin B did not modulate Kiss-1 gene expression (Fig. 1C). Similar to the effect of inhibin A, follistatin stimulation significantly reduced Kiss-1 expression compared to non-stimulated cells (Fig. 1D).

3.2. Expression of inhibin α, βA, and βB subunits and follistatin in primary cultures of fetal rat brain

Quantitative real-time PCR analysis showed that inhibin α, βA, and βB subunit mRNAs were expressed in these neuronal cultures (Fig. 2A). Their protein expression was also detected (Fig. 2B). Furthermore, follistatin mRNA and protein were also expressed (Fig. 2C and D). Tissues from rat ovary and pituitary gland were used as a positive control.
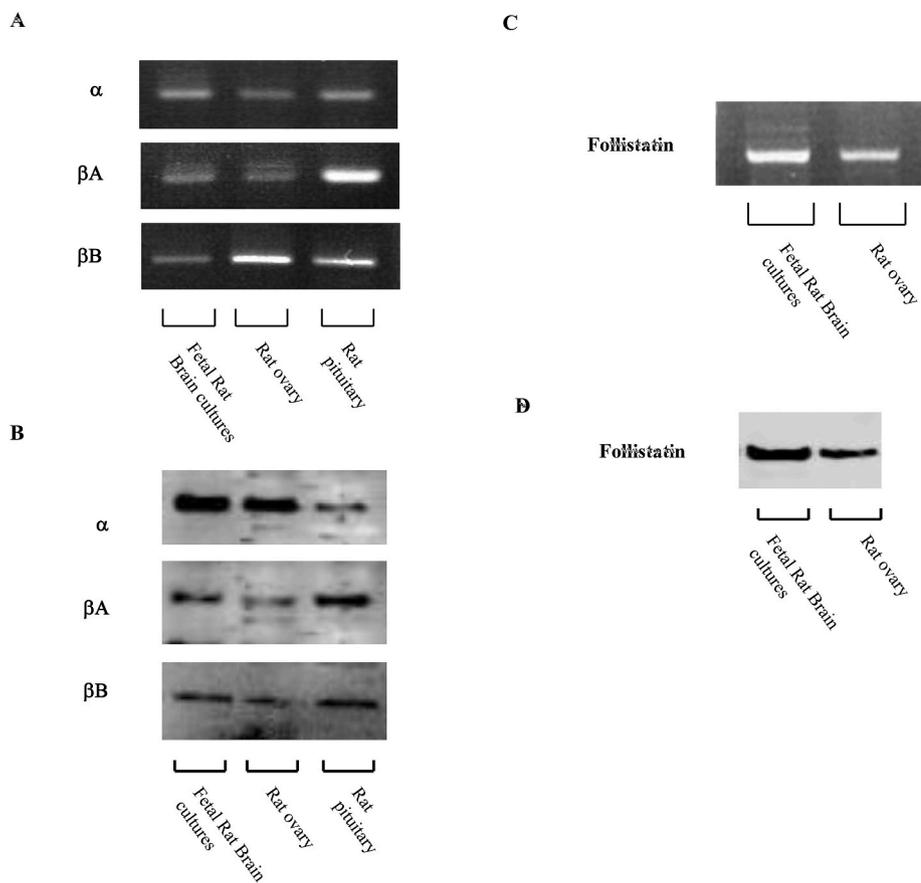
3.3. Effect of E2 on the expression of inhibin α, βA, and βB subunits and follistatin in primary cultures of fetal rat brain

Inhibin α subunit expression was significantly increased by 2.14 ± 0.39-fold by stimulation with 100 nM E2 (Fig. 3A). Similarly, treatment with 100 nM E2 increased the expression of inhibin βA and βB subunits by 2.24 ± 0.48- and 2.54 ± 0.63-fold, respectively (Fig. 3B and C). E2 stimulation at 100 nM also significantly increased follistatin gene expression by 2.97 ± 0.87-fold in these cultures (Fig. 3C).

4. Discussion

In our previous study using a hypothalamic Kiss-1-expressing cell model, mHypoA-55 cells, we reported that exogenous activin increased Kiss-1 expression, whereas inhibin A and follistatin decreased its expression [13]. Our present study confirmed that these gonadal factors had similar effects on Kiss-1 gene expression when we used a more physiological neuronal cell model obtained from fetal rat brain. In these primary neuronal cultures, activin increased the expression of Kiss-1; in contrast, inhibin A and follistatin had an inhibitory effect on Kiss-1 gene expression. These results indicated that activin, inhibin, and follistatin, which are known to play roles in the regulation of follicular development within the ovary [6] and FSH secretion from the pituitary gland [7], also control Kiss-1 gene expression in the brain. Furthermore, the expression levels of genes encoding activin/inhibin and follistatin were increased by E2 stimulation in primary neuronal cultures of fetal rats. Considering these observations, we speculated that activin, inhibin, and follistatin, which are produced in the brain, might be involved in the E2-induced feedback control of the HPG axis.

We detected inhibin subunits (α, βA, and βB) in our neuronal culture,

Fig. 1. Effects of activin, inhibin, and follistatin on Kiss-1 gene expression in primary cultures of fetal rat brain. Neuronal cells obtained from fetal rat brain were stimulated with 10 ng/mL activin A (A), inhibin A (B), inhibin B (C), and follistatin (D) for 24 h mRNA was then extracted and reverse transcribed and Kiss-1 mRNA levels were measured by quantitative real-time PCR. The results are expressed as 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.

A

B

C

D

Fig. 2. Expression of inhibin subunits and follistatin in primary cultures of fetal rat brain. Total RNA was prepared and quantitative real-time PCR was carried out for 40 cycles using primers specific for inhibin α, βB, and βA subunits (A) and follistatin (C). PCR products were resolved in a 1.5% agarose gel and visualized with ethidium bromide staining. Cell lysates (30 μg protein) from primary cultures of fetal rat brain were analyzed by SDS-PAGE followed by immunoblotting and incubation with antibodies against inhibin α, βB, and βA subunits (B) and follistatin (D). The bands were visualized using a horseradish peroxidase-conjugated secondary antibody.

A

B

C

D
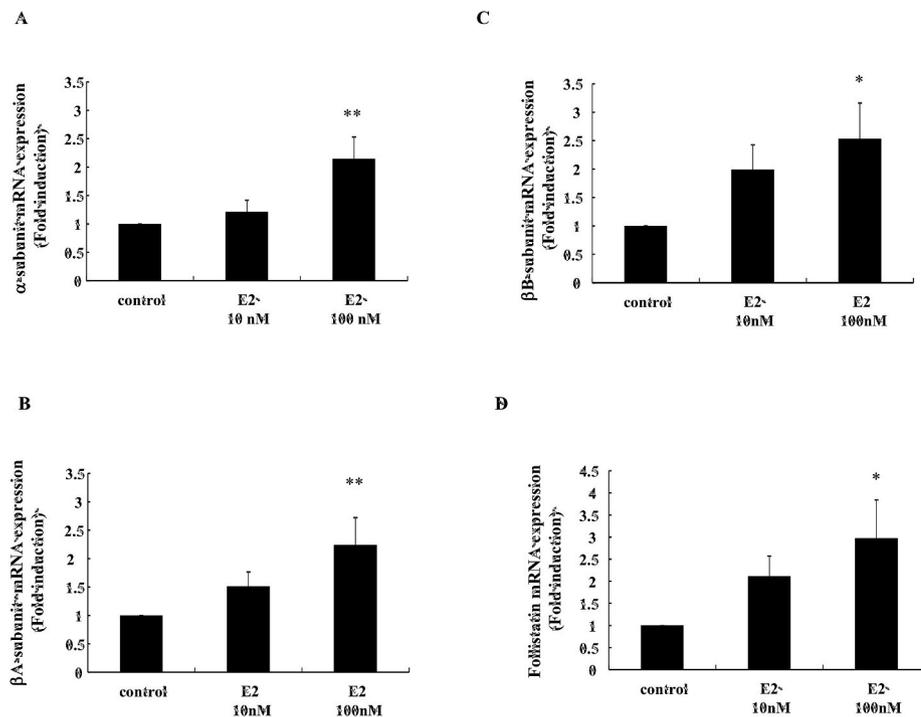
Fig. 3. Effects of E2 on the expression of inhibin subunits and follistatin in primary cultures of fetal rat brain. Neuronal cells obtained from fetal rat brain were stimulated with 10 nM and 100 nM E2 for 48 h, after which mRNA was extracted and reverse transcribed. The mRNA levels of inhibin α (A), βA (B), and βB (C) subunits and follistatin (D) were measured by quantitative real-time PCR. The results are expressed as 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.

suggesting that activin A, B, and AB and inhibin A and B could be expressed at the protein level in the brain. Although we did not examine the detailed distribution of these peptides in whole brain tissues, previous studies reported that these subunits as well as follistatin were expressed in the spinal cord, cerebrum, cerebellum [10], and hypothalamus [17–19]. Activin expression is induced in the brain and acts as a neuroprotective factor after injury or stroke [20,21]. It also plays a role in neuronal development and the differentiation of neuronal stem cells

[22,23]. Furthermore, activin signaling is involved in anxiety behavior and memory [24,25]. These observations suggest that the activin produced within the brain has a role in several neurological processes. Conversely, inhibin and follistatin, which are also expressed in the brain, are considered to reverse the effects of activin.

We found that activin increased Kiss-1 expression, whereas inhibin A and follistatin repressed its expression in primary neuronal cultures. These observations suggested that the Kiss-1 gene, a principal regulator of the HPG axis, is under the control of activin, inhibin, and follistatin. As these gonadal peptides are expressed within the brain, it is plausible that they are produced or induced control Kiss-1, thus participate in the control of the HPG axis. Indeed, a previous *in vivo* study demonstrated that the intracerebroventricular administration of activin significantly increased gonadotropin secretion in male rats [17]. It is well documented that Kiss-1 neurons are located in the AVPV and ARC of the hypothalamus, where they are believed to be involved in E2-induced positive and negative feedback control systems, respectively [2]. Because we used primary cultures of fetal rat brain in this study, we could not distinguish from which region the Kiss-1 neurons responding to activin, inhibin, and follistatin came from. Using rat brain, Takumi et al. (2011) demonstrated that Kiss-1 neurons appeared earlier in the ARC than in the AVPV. In the AVPV, Kiss-1 gene expression was detected at postnatal week 3 in female rats [26]. Considering this report, it is possible that Kiss-1 neurons from the AVPV region of the hypothalamus were absent from our primary neuronal cells, and that the phenomena observed in this study occurred only in Kiss-1 neurons in the ARC region of the hypothalamus. In this regard, we reported previously that activin, inhibin A, and follistatin stimulation do not modify Kiss-1 expression in the mHypoA-50 cell model, which originated from hypothalamic neurons in the AVPV region [13].

It is plausible that activin and follistatin act in an autocrine-paracrine manner within limited areas such as the ovary and anterior pituitary gland. In contrast, the serum levels of inhibin A and B change during the human menstrual cycle. The levels of inhibin B are raised in the early follicular phase, but then fall at the late follicular phase. After the short-lived period after the surge of luteinizing hormone, the concentration of inhibin B falls to a low level during the luteal phase. Inhibin A concentration is low during the follicular phase, but it rises at ovulation, is maintained at a high level during the midluteal phase, and then falls [27]. As for FSH synthesis and secretion, locally produced activin and follistatin act as autocrine-paracrine factors to regulate FSH levels within the pituitary gland [28,29]. In contrast, inhibin produced by the gonads could act at the pituitary gland and inhibit FSH synthesis [27]. At present, we speculate that activin, inhibin, and follistatin expressed within the brain could control Kiss-1 expression, but it is also possible that the effect of activin on Kiss-1 expression is hampered by circulating inhibin A produced by the gonads.

The expression of inhibin subunits, which compose activins/inhibins as well as follistatin, in our neuronal cultures was under the control of E2, indicating that these peptides induced within the brain might participate in the E2-induced feedback control of the HPG axis. At present, the upstream regulator of GnRH-expressing neurons is believed to be Kiss-1 neurons located in the ARC and AVPV [1]. Estrogen receptors are expressed on the surface of these Kiss-1 neurons, and Kiss-1 expression in the ARC is repressed by E2, whereas AVPV Kiss-1 expression is induced by E2 [3]. Our observations that activin could induce Kiss-1 expression and that E2 could induce intracerebral activin expression indicate the possibility that activin is positioned upstream of Kiss-1 neurons and plays some role in the induction of Kiss-1 expression under the influence of E2. Similarly, inhibin and follistatin, both of which are positively regulated by E2, could repress Kiss-1 expression, suggesting that they are positioned upstream of Kiss-1 neurons and might be involved in the E2-induced negative feedback mechanism.

It is obvious that Kiss-1 neurons play pivotal roles to intercede with E2 sufficiency or insufficiency in GnRH neurons; however, we speculate that intracerebral activin, inhibin, and follistatin may also mediate E2 sufficiency in the HPG axis.

Declaration of competing interest

Acknowledgement

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