

Potential role of kisspeptin in the estradiol-induced modulation of inhibin subunit gene expression: Insights from *in vivo* rat models and hypothalamic cell models

Tuvshintugs Tumurbaatar, Haruhiko Kanasaki , Zhuoma Cairang, Batjargal Lkhagvajav, Aki Oride, Hiroe Okada and Satoru Kyo

Department of Obstetrics and Gynecology, Shimane University Faculty of Medicine, Izumo 693-8501, Japan

Abstract. The hypothalamic-pituitary-gonadal (HPG) axis is primarily regulated by kisspeptin neurons. In addition, activin and inhibin within the central nervous system might contribute to the regulation of the HPG axis because they are expressed near kisspeptin and gonadotropin-releasing hormone (GnRH) neurons. We investigated the effects of inhibin and activin within the hypothalamus in the estradiol (E2)-induced negative feedback mechanism. Inhibin α subunit gene within the posterior hypothalamus in female rats increased after ovariectomy, and this increase was completely suppressed by E2 supplementation. In contrast, inhibin β A subunit decreased after ovariectomy and this reduction was recovered by E2. In ovary-intact rats, E2 reduced inhibin α subunit and increased inhibin β A expression within the hypothalamus. In the rHypoE8 and GT1-7 hypothalamic cell models, E2 stimulation increased inhibin α subunit gene expression. Activin and inhibin A increased *Kiss1* gene expression in GT1-7 cells, while inhibin B reduced it. Kisspeptin increased inhibin α subunit expression in rHypoE8 cells, GT1-7 cells, and the mHypoA55 hypothalamic KNDy neuron cell model. Our findings suggest that the expression of inhibin subunits, especially inhibin α , could be increased by E2 in hypothalamic cells and that kisspeptin, inhibin, and activin mutually influence each other under the actions of E2, but their regulation might be controlled mainly by kisspeptin neurons *in vivo*. Although the effects of activin and inhibin on *Kiss1* gene expression varied depending on the hypothalamic cell model examined, intracerebral inhibin and activin might have potential roles in the E2-induced negative feedback mechanism under the influence of kisspeptin neurons.

Key words: Hypothalamic-pituitary gonadal axis, Kisspeptin, Activin, Inhibin, Estradiol

Introduction

Female reproductive function is regulated by the hypothalamic-pituitary-gonadal (HPG) axis. The discovery of the loss of function of the gene encoding the receptor for kisspeptin, a peptide in the hypothalamus, has greatly enhanced our understanding of how the brain controls reproduction [1, 2]. It is now widely recognized that kisspeptin produced in the hypothalamus plays a crucial role in the control of female reproductive systems [3].

Kisspeptin neurons, which are predominantly located in the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) regions of the hypothalamus, project to gonadotropin-releasing hormone (GnRH) neurons and modulate their activity [4, 5]. Kisspeptin neurons in

the AVPV and ARC regions are involved in estradiol (E2)-induced positive and negative feedback mechanisms. This is evidenced by the observations that *Kiss1* gene expression in the AVPV is upregulated by E2, while its expression in the ARC is suppressed by E2 [5, 6]. KNDy neurons within the ARC express kisspeptin, neurokinin B, and dynorphin. Kisspeptin-expressing KNDy neurons produce synchronized oscillatory activity patterns through autocrine excitatory and inhibitory signals from neurokinin B and dynorphin [7, 8].

The discovery of inhibin began in 1923 when Mottram and colleagues reported that irradiation of the testes caused pituitary hypertrophy and obesity in rats; they suggested that a gonadal factor affects pituitary cells [9]. This gonadal hormone was later named “inhibin” due to its ability to inhibit follicle-stimulating hormone (FSH) release from pituitary gonadotroph cells. Inhibin belongs to the transforming growth factor-beta superfamily and is composed of α and β subunits linked by a disulfide bridge paired with an isoform of the β subunit, either a β A or β B subunit, resulting in inhibin A (α/β A) or inhibin B (α/β B),

Submitted Jan. 30, 2025; Accepted Apr. 21, 2025 as EJ25-0044

Released online in J-STAGE as advance publication May 23, 2025

Correspondence to: Haruhiko Kanasaki, MD, PhD, Department of Obstetrics and Gynecology, Faculty of Medicine, Shimane University, 89-1 Enya Cho, Izumo, Shimane 693-8501, Japan.

E-mail: kanasaki@med.shimane-u.ac.jp

respectively. In the ovary, inhibins are produced by granulosa cells of the follicle and play a crucial role in regulating folliculogenesis [10], steroidogenesis [11], and follicular dominance during the preovulatory phase of the menstrual cycle [12]. In addition, gonadal inhibin travels through the bloodstream to act on the pituitary gland, inhibiting FSH release from pituitary gonadotroph cells [13].

Activin was discovered during the isolation of inhibin from porcine ovarian follicular fluid, and was recognized for its role in inducing FSH release, akin to the inhibitory action of inhibin, and was therefore named “activin” [14, 15]. Activin consists of homodimers or heterodimers of β subunits such as activin A ($\beta\text{A}/\beta\text{A}$), activin B ($\beta\text{B}/\beta\text{B}$), and activin AB ($\beta\text{A}/\beta\text{B}$). In the ovary, activin acts as an autocrine/paracrine mediator with various functions such as regulating the early stage of follicle growth and enhancing FSH receptor expression and responsiveness in granulosa cells [15, 16]. Activin is also produced within the pituitary gland, and acts locally to stimulate FSH release from pituitary gonadotroph cells and also regulates FSH β biosynthesis [17, 18]. At present, it is believed that although inhibin produced by the gonads reaches the pituitary gland and reduces FSH expression, only locally produced activin, and not gonadal activin, increases FSH synthesis in the pituitary gland [17, 19–21]. Moreover, gene and protein expression of inhibin and activin subunits has been detected ubiquitously in the human central nervous system (CNS), including the hypothalamus, although the neuronal or non-neuronal cells that specifically express these subunits have not been definitively identified in the hypothalamus [22, 23].

Previously, we demonstrated that activin significantly increases *Kiss1* gene expression in the mHypoA55 KNDy neuron hypothalamic cell model, while inhibin A (but not inhibin B) decreases *Kiss1* expression. In addition, we found that inhibin α subunit gene expression is increased by E2 stimulation in mHypoA55 cells [24]. Furthermore, similar results were obtained using primary cultures of fetal rat neuronal cells, in which exogenous activin stimulation increases *Kiss1* gene expression, while inhibin A decreases it. Finally, the expression of inhibin subunits in these neuronal cultures is increased by E2 [25]. Our previous studies suggest the possibility that activin and inhibin expressed in the CNS play crucial roles in the regulation of *Kiss1* gene expression under the influence of E2.

To examine the possible involvement of hypothalamic activin and inhibin in the E2-induced negative feedback mechanism, we used an *in vivo* animal model to investigate the effect of ovariectomy (OVX) on inhibin subunit expression in the hypothalamus. In addition, we examined the effect of E2 on inhibin subunit gene expression and the relationship between kisspeptin and activin/inhibin

expression in an *in vitro* study using hypothalamic cell models originating from different hypothalamic areas to determine the regulatory mechanism of activin and inhibin in the CNS.

Materials and Methods

Materials

The following chemicals and reagents were obtained from the indicated sources: fetal bovine serum (Invitrogen, Carlsbad, CA); Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, and water-soluble E2 (Sigma-Aldrich Co., St. Louis, MO); activin A and inhibin B (Abcam, Cambridge, MA); inhibin A (R&D Systems, Minneapolis, MN); and mouse kisspeptin-10 (KP10; AnaSpec, Fremont, CA).

Animal experiments

Six-week-old female Wistar rats were maintained under a 12-h light/dark cycle at 20–25°C with food (CE-2; CLEA Japan, Tokyo, Japan) and water available *ad libitum*. Vaginal smears were taken daily, and only rats showing at least three consecutive regular estrus cycles were included in this study. The rats received OVX under intraperitoneal anesthesia with an injection of medetomidine (0.15 mg/kg), midazolam (2 mg/kg), and butorphanol (2.5 mg/kg). Seven days later, a 17 β -E2 (0.25 mg, 21-day release time; Innovative Research of America, Sarasota, FL) or placebo pellet was implanted subcutaneously under intraperitoneal anesthesia. Then, the rats were bred for an additional 7 days. The rats were euthanized under isoflurane anesthesia, and the whole brain was extracted and divided equally in the sagittal plane. One half of the brain was preserved for future experiments, while the other half was used for the present study. The hypothalamus was dissected with reference to an anatomical rat brain atlas [26]. Then, the hypothalamus was divided coronally into anterior and posterior parts for further experimental analysis. This protocol was approved by the Ethics Committee of the Experimental Animal Center for Integrated Research at Shimane University (IZ4-10-2).

Cell culture

The rHypoE8 R8 embryonic rat hypothalamic cell line was purchased from COSMO Bio Co., Ltd. (Tokyo, Japan). The GT1-7 mouse GnRH-producing hypothalamic cell line was kindly provided by Dr. P. Mellon (University of California, San Diego, CA). The mHypoA55 ARC region hypothalamus-derived KNDy neuron model was purchased from Cedarlane (Burlington, Canada). We selected these cell models to investigate the differential regulation of inhibin subunits as well as *Kiss1* gene expression in cells from different regions of the hypothalamus. The

cells were plated in 35-mm tissue culture dishes and incubated with high-glucose DMEM containing 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin at 37°C under a humidified atmosphere of 5% CO₂ in air. After 48 h, the culture medium was changed to high-glucose DMEM containing 1% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin and incubated without (control) or with the test reagents for the indicated periods.

Western blot analysis

Cell extracts were lysed on ice with RIPA buffer (phosphate-buffered saline, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) containing 0.1 mg/mL phenylmethyl sulfonyl fluoride, 30 mg/mL aprotinin, and 1 mM sodium orthovanadate, scraped for 20 s, boiled for 10 min at 100°C, and centrifuged at 15,000 rpm for 10 min at 4°C. Protein concentration in the cell lysates was measured using the Bradford method. Denatured protein (10 µg) was resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis according to standard protocols. Protein was transferred onto polyvinylidene difluoride membranes (Hybond-P PVDF; Amersham Biosciences, Little Chalfont, UK), which were blocked for 1 h at room temperature in Blotto (5% milk in Tris-buffered saline), including an anti-kisspeptin antibody (1:100 dilution; Abcam) [27], anti-inhibin α antibody (1:100 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX) [28], anti-inhibin β A antibody (1:100 dilution; Santa Cruz Biotechnology, Inc.) [29], anti-inhibin β B antibody (1:1,000 dilution; Abcam) [30], or anti- β -actin primary antibody (1:1,000 dilution; Abcam) [31] in Blotto overnight at 4°C and washed three times for 5 min per wash with Tris-buffered saline/1% Tween. Subsequent incubation with a horseradish peroxidase-conjugated antibody was performed for 1 h at room temperature in Blotto, and additional washes were performed as needed. Following enhanced chemiluminescence detection (Amersham Biosciences), membranes were exposed to X-ray film (Fujifilm, Tokyo, Japan). Tissue from rat cerebral cortex was used as a positive control.

RNA preparation, reverse transcription, PCR, and quantitative real-time (RT) PCR

Total RNA was extracted from rat posterior hypothalamus tissue or rHypoE8, GT1-7, and mHypoA55 cells using TRIzol-LS (Molecular Research Center, Inc., Cincinnati, OH). 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) and 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 (#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 , inhibin β A, inhibin β B, and *Kiss1* mRNAs, after PCR amplification using primers for inhibin α (forward: 5'-GTGGGGAGGTCCTAGACAG A-3'; reverse: 5'-GTGGGGATGGCCGGAATACA-3'), inhibin β A (forward: 5'-GGAGTGGATGGCAAGGTC AAC-3'; reverse: 5'-GTGGGCACACAGCATGACTT A-3'), inhibin β B (forward: 5'-GGTCCGCCTGTACTTC TTCGTCT-3'; reverse: 5'-GGTATGCCAGCCGCTACG TT-3'), and *Kiss1* (forward: 5'-ATGATCTCGCTGGCTT CTTGG-3'; reverse: 5'-GGTTCACCACAGGTGCCATT TT-3'), amplicons were electrophoresed in agarose gels and visualized with ethidium bromide staining. cDNA from rat brain was used as a positive control. Quantification of inhibin α , β A, and β B, and *Kiss1* expression was performed by quantitative RT-PCR (Takara TP900; Takara-Bio, Tokyo, Japan) using Universal ProbeLibrary Probes and FastStart Master Mix (Roche Diagnostics, Mannheim, Germany). The PCR primers were designed based on the published sequences of inhibin α , β A, and β B, and *Kiss1* [24]. The simultaneous measurement of target mRNAs and GAPDH permitted the normalization of transcript levels. Each set of primers included a no-template control. The thermal cycling conditions were as follows: 10 min of denaturation at 94°C, followed by 40 cycles of 94°C for 15 s and 55°C for 1 min. Reactions were followed by melting curve analysis (55°C–95°C). To determine PCR efficiency, 10-fold serial dilutions of cDNA were used as previously described [32]. PCR conditions were optimized to obtain >95% efficiency, and only those reactions with efficiencies between 95% and 105% were included in subsequent analyses. Relative differences in cDNA concentration between baseline and experimental conditions were calculated using the comparative threshold cycle (Ct) method [33]. Briefly, for each sample, Δ Ct was calculated for normalization against the internal control using the following equation: Δ Ct = Ct (gene) – Ct (GAPDH). To obtain differences between experimental and control conditions, $\Delta\Delta$ Ct was calculated as Δ Ct (sample) – Δ Ct (control). Relative mRNA levels were 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 mRNA expression was determined, two samples were assayed in duplicate. Six averages from three independent experiments were statistically analyzed. Data are expressed as the

mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance with Bonferroni's *post hoc* test or Student's *t*-test as appropriate. $p < 0.05$ was considered statistically significant.

Results

Effect of OVX and E2 supplementation on inhibin subunit gene expression in the hypothalamus of female rats

The impact of OVX on inhibin subunit mRNA expression within the hypothalamus of female rats was investigated. Following OVX, inhibin α subunit gene expression was significantly increased by 1.72 ± 0.18 -fold, but this increase was completely inhibited by E2 supplementation (Fig. 1A). In contrast, inhibin βA subunit gene expression was decreased after OVX by 0.69 ± 0.03 -fold, but this decrease was repressed by E2 supplementation after OVX (Fig. 1B). Inhibin βB subunit gene expression remained unchanged by OVX and OVX + E2 supplementation (Fig. 1C). In the hypothalamus of ovary-intact rats, inhibin α subunit gene expression was decreased by 0.75 ± 0.10 -fold by E2 administration (Fig. 1D). In contrast, inhibin βA subunit gene expression within the hypothalamus was increased by 1.96 ± 0.49 -fold following E2 administration in ovary-intact rats (Fig. 1E). Inhibin βB subunit gene expression was not modulated

by E2 administration in these rats (Fig. 1F).

Kiss1 and inhibin α , βA , and βB expression in hypothalamic cell models

We previously demonstrated that the inhibin α , βA , and βB subunits are expressed in the mHypoA55 hypothalamic KNDy neuron cell model as well as rat brain neuronal cells [24, 25]. In the present study, we used two hypothalamic cell models, rHypoE8 and GT1-7. As observed in rat brain neuronal cells, all inhibin subunit genes as well as the *Kiss1* gene (Fig. 2A) and their proteins (Fig. 2B) were expressed in rHypoE8 and GT1-7 cells.

Effect of E2 on inhibin subunit gene expression in hypothalamic cell models

Changes in the gene expression of inhibin subunits after E2 stimulation in rHypoE8 and GT1-7 cells were examined. Inhibin α subunit gene expression was significantly increased by E2 stimulation in both cell models. In rHypoE8 cells, inhibin α subunit gene expression was significantly increased by 2.25 ± 0.17 -fold and 2.96 ± 0.02 -fold by 10 and 100 nM E2, respectively (Fig. 3A). Similarly, 100 nM E2 increased inhibin α subunit gene expression in GT1-7 cells by 3.24 ± 0.88 -fold (Fig. 3D). Inhibin βA and βB subunit gene expression was not regulated by E2 treatment in either rHypoE8 or GT1-7 cells (Fig. 3B, C, E, F).

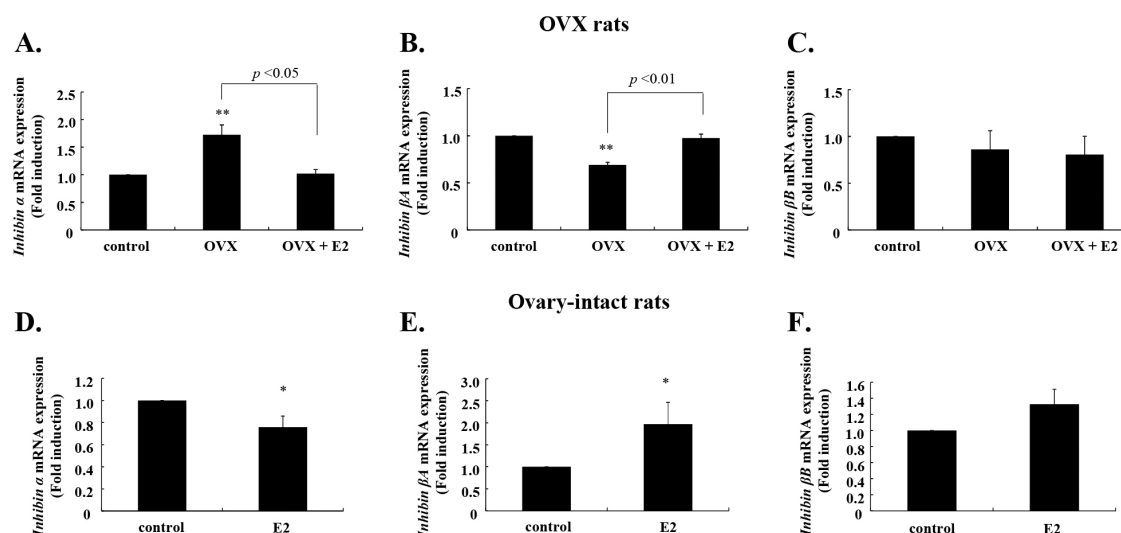


Fig. 1 Effect of OVX and E2 supplementation on inhibin subunit gene expression in female rats.

Six-week-old female rats received OVX or a 0.25 mg E2 pellet was implanted subcutaneously after OVX (OVX + E2). Sham-operated (ovary-intact) rats were used as a control (A–C). In another series of experiments, a 0.25 mg E2 pellet was implanted subcutaneously in ovary-intact 6-week-old female rats. Sham-operated (no E2 pellet) rats were used as a control (D–F). Seven days later, the rats were euthanized, and the posterior part of the hypothalamus was removed. mRNA was extracted from the hypothalamic tissues and reverse transcribed. The mRNA levels of inhibin α (A and D), βA (B and E), and βB (C and F) were measured by quantitative RT-PCR. Samples for each experimental group were run in duplicate and normalized to the mRNA levels of GAPDH as a housekeeping gene. The results are expressed as fold induction over control and presented as the mean $\pm$ SEM. ** $p < 0.01$, * $p < 0.05$ vs. control.

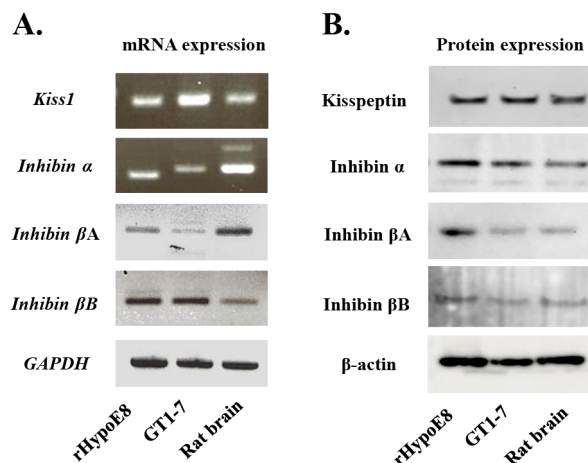


Fig. 2 Expression of *Kiss1* and inhibin α , β A, and β B genes and their proteins in hypothalamic cell models.

(A) Total RNA was extracted from rHypoE8 and GT1-7 cells, and RT-PCR was carried out for 40 cycles using primers specific to *Kiss1*, inhibin α , inhibin β A, and inhibin β B. PCR products were resolved in a 1.5% agarose gel and visualized with ethidium bromide staining. mRNA from rat brain tissues was used as a positive control. (B) Cell lysates (10 μ g protein) from rHypoE8 and GT1-7 cells were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by immunoblotting and incubation with antibodies against kisspeptin, inhibin α , inhibin β A, and inhibin β B. β -Actin was detected as an internal control. Proteins from rat brain tissues were used as a positive control. The bands were visualized using a horseradish peroxidase-conjugated secondary antibody.

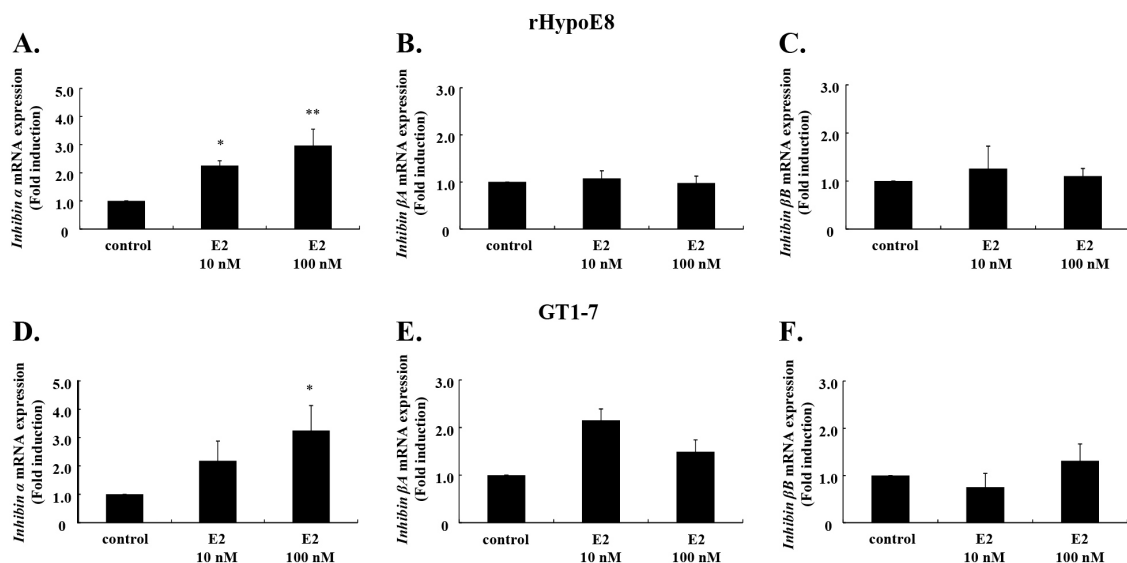


Fig. 3 Effect of E2 on inhibin subunit gene expression in hypothalamic cell models.

rHypoE8 (A–C) and GT1-7 (D–F) cells were stimulated with 10 or 100 nM E2 for 24 h, after which mRNA was extracted and reverse transcribed. Inhibin α (A and D), β A (B and E), and β B (C and F) subunit mRNA levels were measured by quantitative RT-PCR. Results are expressed as fold induction over unstimulated cells and presented as mean $\pm$ SEM values of three independent experiments, each performed with duplicate samples. ** $p < 0.01$, * $p < 0.05$ vs. control.

Effect of activin and inhibin A and B on *Kiss1* gene expression in hypothalamic cell models

Next, we examined the effects of activin and inhibin on *Kiss1* gene expression in the rHypoE8 and GT1-7 cell lines. In rHypoE8 cells, stimulation with activin, inhibin A, or inhibin B did not modulate *Kiss1* gene expression (Fig. 4A–C). However, in GT1-7 cells, 10 ng/mL activin

significantly increased *Kiss1* gene expression by 1.4 ± 0.10 -fold (Fig. 4D). In addition, 10 ng/mL inhibin A significantly increased *Kiss1* gene expression in GT1-7 cells by 2.17 ± 0.22 -fold (Fig. 4E). However, 10 ng/mL inhibin B significantly decreased *Kiss1* gene expression by 0.57 ± 0.07 -fold (Fig. 4F).

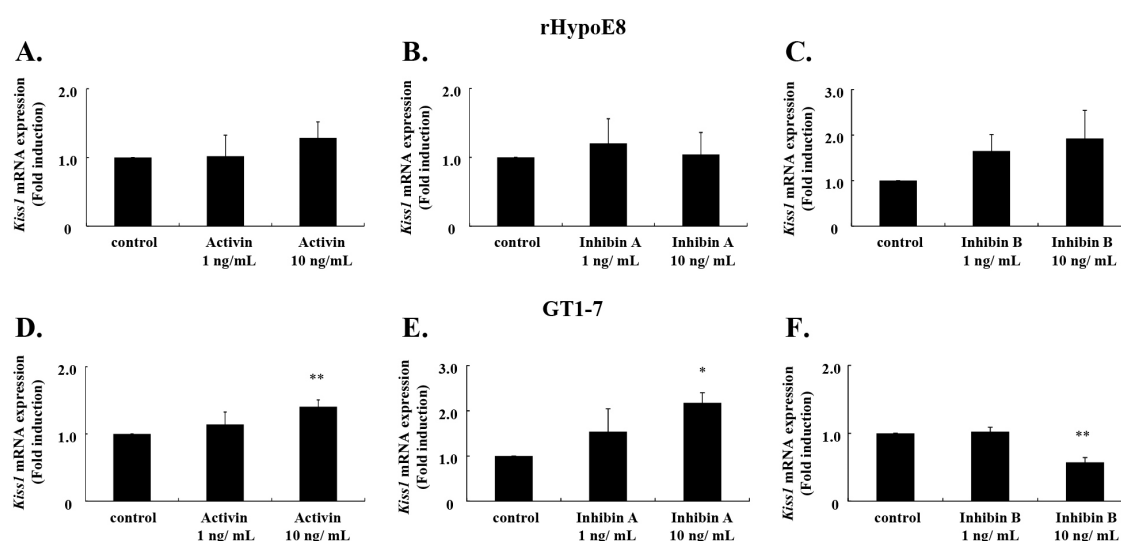


Fig. 4 Effect of activin, inhibin A, and inhibin B on *KissI* gene expression in hypothalamic cell models.

rHypoE8 (A–C) and GT1-7 (D–F) hypothalamic cell models were stimulated with the indicated concentrations of activin (A and D), inhibin A (B and E), or inhibin B (C and F) for 24 h. After which, mRNA was extracted and reverse transcribed. *KissI* mRNA levels were measured by quantitative RT-PCR. Results are expressed as fold induction over unstimulated cells and presented as mean $\pm$ SEM values of three independent experiments, each performed with duplicate samples. ** $p < 0.01$, * $p < 0.05$ vs. control.

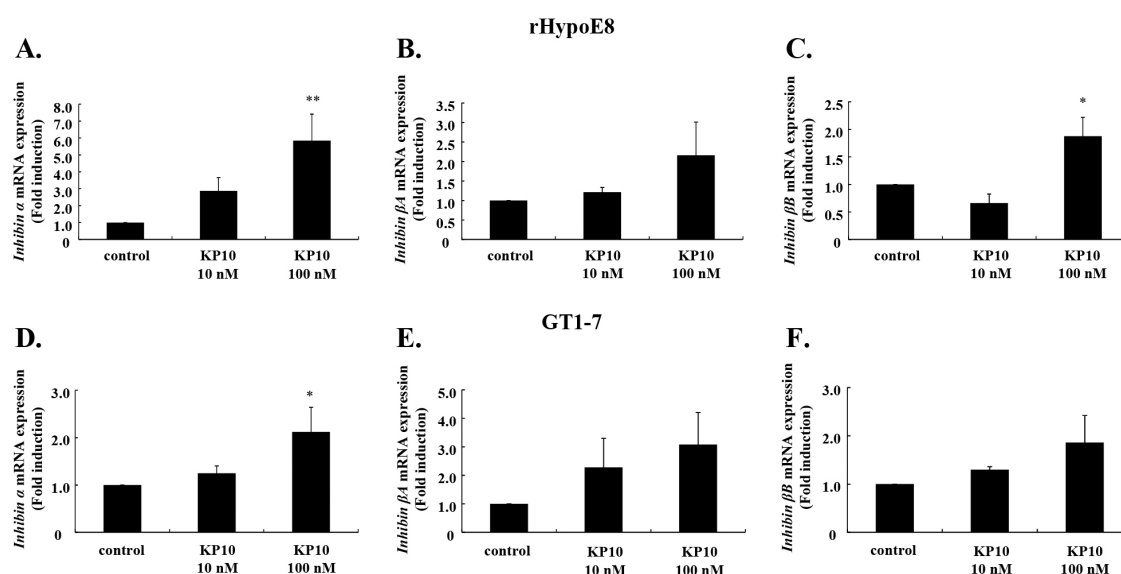


Fig. 5 Effect of KP10 on inhibin subunit gene expression in hypothalamic cell models.

rHypoE8 (A–C) and GT1-7 (D–F) hypothalamic cell models were stimulated with the indicated concentrations of KP10 for 24 h. After which, mRNA was extracted and reverse transcribed. Inhibin α (A and D), βA (B and E), and βB (C and F) subunit mRNA levels were measured by quantitative RT-PCR. Results are expressed as fold induction over unstimulated cells and presented as mean $\pm$ SEM values of three independent experiments, each performed with duplicate samples. ** $p < 0.01$, * $p < 0.05$ vs. control.

Effect of KP10 on inhibin subunit gene expression in hypothalamic cell models

Next, we examined the effect of KP10 on inhibin subunit gene expression in hypothalamic cell models. In rHypoE8 cells, inhibin α subunit gene expression was significantly increased by 5.83 ± 1.57 -fold following stimulation with 100 nM KP10 (Fig. 5A). KP10 did not have a significant effect on βA subunit expression, but we

observed a slight increase in inhibin βB subunit levels in these cells (1.88 ± 0.34 -fold) (Fig. 5B, C). Similarly, 100 nM KP10 significantly increased inhibin α subunit gene expression in GT1-7 cells by 2.02 ± 0.52 -fold (Fig. 5D). However, KP10 did not have a significant effect on inhibin βA and βB subunit gene expression in GT1-7 cells (Fig. 5E, F).

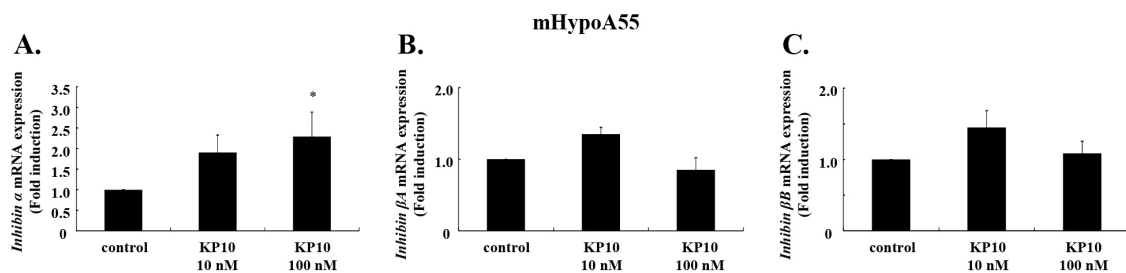


Fig. 6 Effect of KP10 on inhibin subunit gene expression in the mHypoA55 KNDy neuron cell model.

mHypoA55 cells were stimulated with the indicated concentrations of KP10 for 24 h. After which, mRNA was extracted and reverse transcribed. Inhibin α (A), βA (B), and βB (C) subunit mRNA levels were measured by quantitative RT-PCR. Results are expressed as fold induction over unstimulated cells and presented as mean $\pm$ SEM values of three independent experiments, each performed with duplicate samples. * $p < 0.05$ vs. control.

Effect of KP10 on inhibin subunit gene expression in the mHypoA55 KNDy neuron cell model

Female mouse hypothalamus-derived mHypoA55 cells are known as a KNDy neuron cell model. Under certain conditions, E2 inhibits *Kiss1* gene expression in these cells [34]. In addition, we have previously shown that activin increases *Kiss1* gene expression, while inhibin A inhibits *Kiss1* expression in these cells [24]. In this cell model, 100 nM KP10 significantly increased inhibin α subunit expression by 2.29 ± 0.59 -fold (Fig. 6A). However, KP10 treatment did not alter inhibin βA and βB subunit gene expression in mHypoA55 cells (Fig. 6B, C).

Discussion

Inhibin is secreted by the gonads and negatively regulates FSH production in the pituitary gland [17, 35–37], where activin and its receptors are also present [38]. Activin produced by the gonads has no effect on the pituitary gland, but activin produced within the pituitary gland promotes FSH production in pituitary gonadotroph cells in a paracrine manner [19–21, 35, 39]. Furthermore, activin and inhibin are thought to contribute to the HPG axis in the hypothalamus. Although it is still unknown which neuronal or non-neuronal cells in the CNS specifically express inhibin subunits, in humans and rodents, inhibin and activin subunits are found ubiquitously in the CNS including the hypothalamic area in which GnRH neurons are located [22, 23, 40].

We previously demonstrated that the expression levels of the inhibin α , βA , and βB subunits as well as follistatin are distinct in cell models derived from different areas of the hypothalamus, including the mouse mHypoA55 ARC-derived KNDy neuron cell model, mouse mHypoA50 AVPV-derived cell model, and primary cultures of fetal rat brain tissues [24, 25]. Here, we again confirmed that inhibin subunits were expressed in other hypothalamic cell models, namely, rHypoE8 cells (originating from

embryonic rat hypothalamus) and GT1-7 cells (derived from mouse GnRH-producing neurons). This implies that activin and inhibin are expressed in hypothalamic cells.

In our previous study using the mHypoA55 ARC-derived KNDy neuron cell model, we found that activin A increases *Kiss1* gene expression, while inhibin A inhibits its expression. Conversely, in mHypoA50 cells, which originate from the AVPV region of the hypothalamus, neither activin A nor inhibin A alters *Kiss1* gene expression [24]. Since activin and inhibin only had effects on the KNDy neuron cell model, we aimed to elucidate the regulatory mechanisms underlying the E2-induced negative feedback mechanism and possible involvement of activin and inhibin expressed in the hypothalamus in this process. First, we conducted *in vivo* experiments examining inhibin subunit expression within the posterior part of the hypothalamus in OVX rats and ovary-intact rats after E2 treatment to observe the direct effect of E2 on inhibin subunit expression in the hypothalamus *in vivo*. The posterior hypothalamic region was extracted because it contains the ARC region, which is an E2-induced negative feedback center [6, 26]. In addition, we employed the rHypoE8, GT1-7, and mHypoA55 cell lines to investigate the cellular response to E2, activin, inhibin, and KP10 *in vitro* by monitoring inhibin subunit and *Kiss1* gene expression. Inhibin α subunit gene expression in the rat posterior hypothalamus was increased by OVX, and this effect was inhibited by E2 supplementation after OVX, indicating that E2 negatively regulates inhibin α subunit gene expression in the hypothalamus. The observation that E2 supplementation repressed inhibin α subunit gene expression in ovary-intact rats supports this hypothesis. In contrast, inhibin βA subunit gene expression decreased following OVX, but this reduction was repressed with E2 supplementation. Additionally, E2 supplementation increased inhibin βA subunit gene expression in ovary-intact rats. The impact of E2 on the inhibin βA subunit differed from its effect on the inhibin α

subunit. Thus, it is plausible that the inhibin subunit genes expressed in the hypothalamus are regulated differently by the E2 milieu *in vivo*.

The inhibin subunits, especially the α subunit within the hypothalamus, seem to be strongly influenced by E2. In *in vitro* experiments using the rHypoE8 and GT1-7 hypothalamic cell models, E2 stimulation strongly upregulated inhibin α subunit gene expression. This observation was quite similar to our previous findings, which showed that E2 induces a significant increase in inhibin α subunit gene expression in the mHypoA55 cell line and fetal rat brain cultures [24, 25]. In contrast, E2 has no significant effect on inhibin β A and β B subunit gene expression in rHypoE8, GT1-7 cells, and mHypoA55 cells [24]. However, E2 has a stimulatory effect on inhibin β A and β B subunit gene expression in fetal rat brain cultures [25]. From these observations, we can speculate that E2 could positively regulate the expression of inhibin subunit genes, particularly the α subunit, in hypothalamic cells. Otherwise, we can say that although inhibin α subunit expression in hypothalamic cells is stimulated by E2, the β A and β B inhibin subunits show different responses to E2 depending on the type of hypothalamic cell.

Although hypothalamic inhibin subunit genes, especially inhibin α , were positively regulated by E2, it was revealed that E2 is not the main regulator of inhibin subunit expression within the brain because *in vivo* observations using OVX rats showed that depletion of E2 significantly increased hypothalamic inhibin α subunit expression and reduced β A subunit expression. If inhibin subunit expression within the hypothalamus is principally regulated by E2, how do inhibin and activin, which are composed of inhibin subunits, transduce E2 signals to kisspeptin neurons, which govern the HPG axis? We previously observed that activin A significantly increases *Kiss1* expression in the mHypoA55 KNDy neuron cell model, whereas inhibin A, but not inhibin B, decreases *Kiss1* expression or antagonizes the activin-induced *Kiss1* gene expression in these cells [24]. In the present study, *Kiss1* gene expression in rHypoE8 cells was not modulated by exogenous activin A or inhibin A/B treatment. In contrast, activin A and inhibin A treatment slightly increased *Kiss1* gene expression in GT1-7 cells, while inhibin B decreased it at higher concentrations. The effect of activin on *Kiss1* gene expression was identical in GT1-7 and mHypoA55 cells, but the effects of inhibin A and inhibin B were distinct in these cells. In addition, in rHypoE8 cells, neither activin nor inhibin altered *Kiss1* expression. Therefore, the effects of activin and inhibin on *Kiss1* gene expression might depend on the type of hypothalamic cell. Activin and inhibin may more clearly exert their effects on *Kiss1* gene expression in KNDy neurons.

In this study, we also examined the effect of KP10 on inhibin subunit gene expression in hypothalamic cell models. Interestingly, exogenous KP10 treatment increased inhibin α subunit expression, but not β A or β B expression, in the rHypoE8 and GT1-7 cell lines. In the mHypoA55 cell line, inhibin α subunit gene expression was also increased by KP10. Although the effects of inhibin or activin on *Kiss1* gene expression were distinct between the mHypoA55 cell line and other hypothalamic rHypoE8 or GT1-7 cells, the effect of KP10 on the inhibin α subunit was identical in all of these cell lines. From the observation of the increased expression of the inhibin α subunit gene by KP10 in all hypothalamic cell models, we now speculate that inhibin α subunit gene expression in the hypothalamus is upregulated by kisspeptin neurons.

It is generally agreed that *Kiss1* gene expression in the ARC region is upregulated in OVX rats and this increase is repressed by E2 and progesterone [4, 5, 8]. This phenomenon was quite similar to that observed for inhibin α subunit gene expression in hypothalamic areas including the ARC. On the basis of the effects of KP10 in the hypothalamic cell models examined here, it is considered that *Kiss1* gene expression is increased in the ARC region of the hypothalamus by OVX, and the increase in kisspeptin affects the neighboring inhibin subunit-expressing neurons and increases α subunit expression in the hypothalamus.

Although the effects of activin and inhibin on *Kiss1* gene expression varied depending on the hypothalamic cell type, activin increased *Kiss1* gene expression in GT1-7 cells and mHypoA55 KNDy neuron cells [24]. However, inhibin A inhibited *Kiss1* gene expression in mHypoA55 cells [24], but it increased it in GT1-7 cells. Furthermore, inhibin B inhibited *Kiss1* gene expression in GT1-7 cells, but not in mHypoA55 cells [24]. These observations indicate that activin and inhibin within the brain have some effects on kisspeptin-expressing neurons including KNDy neurons. However, considering the observations that OVX induced *Kiss1* gene as well as inhibin α subunit expression in the same hypothalamic area and that KP10 increased inhibin α subunit expression in several hypothalamic cell lines, hypothalamic inhibin might be more closely affected by kisspeptin neurons *in vivo* because inhibin is composed of the inhibin α subunit.

A prior *in vivo* investigation revealed that an intracerebroventricular infusion of activin notably elevates the serum levels of luteinizing hormone in adult male rats, while FSH levels are unaffected [40]. This finding suggests a potential interaction between hypothalamic activin and kisspeptin neurons, which govern the HPG axis. In addition, inhibin might reduce the endogenous release of GnRH from the hypothalamus *in vivo* and *in vitro* [41]. Exploring the molecular signaling events in

the hypothalamus poses significant challenges due to the heterogeneous nature of neuronal cell populations, where individual cells express multiple neuropeptides. However, in our study using an OVX rat model, we have revealed some evidence regarding the regulatory mechanisms governing the expression of inhibin subunits, particularly in response to E2 depletion.

Conclusion

Our findings may provide insights about the possible involvement of activin and inhibin expressed in the hypothalamus in the E2-induced negative feedback mechanism in the HPG axis. The expression pattern of the inhibin α subunit gene in the posterior hypothalamus after OVX and sex steroid supplementation after OVX was identical to that observed previously for *Kiss1* gene expression in this area. Inhibin subunits, especially inhibin α , could be positively modulated by E2 in hypothalamic cells, but their expression might be controlled mainly by kisspeptin neurons *in vivo* (Fig. 7, Graphical Abstract). Although the effects of activin and inhibin on *Kiss1* gene expression varied depending on the hypothalamic cell type examined, these differences may simply be due to the characteristics of each cell type. To determine the roles of inhibin and activin in genuine hypothalamic cells in a more physiological setting, we should administer inhibin, activin, or kisspeptin into the hypothalamus and investigate its effects on the HPG axis. Nevertheless, from our current study, we can speculate that although intracerebral inhibin, activin, and kisspeptin mutually influence each other, inhibin and activin expressed in the hypothalamus might have roles in reproductive physiology after E2 depletion under the influence of hypothalamic kisspeptin neurons.

Authors' Contributions

T.T., and H.K. designed the research; T.T., Z.C., B.L., A.O., and H.O. performed the research and analysed data; T.T., and H.K. wrote the paper; S.K. supervised this research.

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.).

Availability of supporting data

The datasets used and/or analyzed during this study

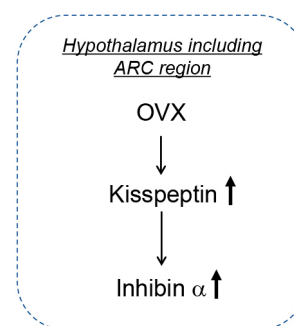
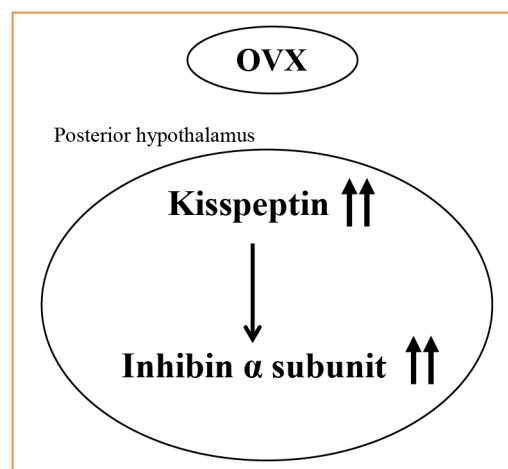


Fig. 7 Possible role of kisspeptin in the OVX-induced modulation of inhibin α subunit expression.

Inhibin α subunit expression in the posterior area of the hypothalamus was increased following OVX, and this increase was suppressed by E2 supplementation. This pattern mirrors the well-established E2-mediated suppression of *Kiss1* gene expression in this region. Because kisspeptin stimulation significantly increased inhibin α subunit expression, and because neither activin nor inhibin modulated *Kiss1* gene expression in a variety of hypothalamic cells, we speculate that kisspeptin may act as an upstream regulator of inhibin and activin expression in the hypothalamus.



Graphical Abstract

are available from corresponding authors upon reasonable request.

Code availability

Not applicable.

Declarations

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Consent for participation

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

- de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, *et al.* (2003) Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci U S A* 100: 10972–10976.
- Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS Jr, *et al.* (2003) The GPR54 gene as a regulator of puberty. *N Engl J Med* 349: 1614–1627.
- Pinilla L, Aguilar E, Dieguez C, Millar RP, Tena-Sempere M (2012) Kisspeptins and reproduction: physiological roles and regulatory mechanisms. *Physiol Rev* 92: 1235–1316.
- Kauffman AS, Gottsch ML, Roa J, Byquist AC, Crown A, *et al.* (2007) Sexual differentiation of Kiss1 gene expression in the brain of the rat. *Endocrinology* 148: 1774–1783.
- Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA (2005) Regulation of Kiss1 gene expression in the brain of the female mouse. *Endocrinology* 146: 3686–3692.
- Adachi S, Yamada S, Takatsu Y, Matsui H, Kinoshita M, *et al.* (2007) Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats. *J Reprod Dev* 53: 367–378.
- Navarro VM, Castellano JM, Fernández-Fernández R, Barreiro ML, Roa J, *et al.* (2004) Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. *Endocrinology* 145: 4565–4574.
- Wakabayashi Y, Nakada T, Murata K, Ohkura S, Mogi K, *et al.* (2010) 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* 30: 3124–3132.
- Mottram JC, Cramer W (1923) On the general effects of exposure to radium on metabolism and tumour growth in the rat and the special effects on testis and pituitary. *Q J Exp Physiol* 13: 209–226.
- Woodruff TK, Lyon RJ, Hansen SE, Rice GC, Mather JP (1990) Inhibin and activin locally regulate rat ovarian folliculogenesis. *Endocrinology* 127: 3196–3205.
- Hsueh AJ, Dahl KD, Vaughan J, Tucker E, Rivier J, *et al.* (1987) Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. *Proc Natl Acad Sci U S A* 84: 5082–5086.
- Hillier SG, Wickings EJ, Illingworth PI, Yong EL, Reichert LE Jr, *et al.* (1991) Control of immunoactive inhibin production by human granulosa cells. *Clin Endocrinol (Oxf)* 35: 71–78.
- Welt CK, Smith PC, Taylor AE (2004) Evidence of early ovarian aging in fragile X premutation carriers. *J Clin Endocrinol Metab* 89: 4569–4574.
- Ling N, Ying SY, Ueno N, Shimasaki S, Esch F, *et al.* (1986) A homodimer of the beta-subunits of inhibin A stimulates the secretion of pituitary follicle stimulating hormone. *Biochem Biophys Res Commun* 138: 1129–1137.
- Ling N, Ying SY, Ueno N, Shimasaki S, Esch F, *et al.* (1986) Pituitary FSH is released by a heterodimer of the beta-subunits from the two forms of inhibin. *Nature* 321: 779–782.
- Knight PG, Satchell L, Glister C (2012) Intra-ovarian roles of activins and inhibins. *Mol Cell Endocrinol* 359: 53–65.
- Bilezikjian LM, Blount AL, Donaldson CJ, Vale WW (2006) Pituitary actions of ligands of the TGF-beta family: activins and inhibins. *Reproduction* 132: 207–215.
- Vale W, Rivier J, Vaughan J, McClintock R, Corrigan A, *et al.* (1986) Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature* 321: 776–779.
- Gray PC, Bilezikjian LM, Vale W (2001) Antagonism of activin by inhibin and inhibin receptors: a functional role for betaglycan-glycan. *Mol Cell Endocrinol* 180: 47–53.
- Makanji Y, Harrison CA, Robertson DM (2011) Feedback regulation by inhibins A and B of the pituitary secretion of follicle-stimulating hormone. *Vitam Horm* 85: 299–321.
- Walton KL, Makanji Y, Robertson DM, Harrison CA (2011) The synthesis and secretion of inhibins. *Vitam Horm* 85: 149–184.
- Miller MC, Lambert-Messerlian GM, Eklund EE, Heath NL, Donahue JE, *et al.* (2012) Expression of inhibin/activin proteins and receptors in the human hypothalamus and basal forebrain. *J Neuroendocrinol* 24: 962–972.
- Tuuri T, Erämaa M, Hildén K, Ritvos O (1994) The tissue distribution of activin beta A- and beta B-subunit and follistatin messenger ribonucleic acids suggests multiple sites of action for the activin-follistatin system during human development. *J Clin Endocrinol Metab* 78: 1521–1524.
- Tumurgan Z, Kanasaki H, Tumurbaatar T, Oride A, Okada H, *et al.* (2019) Role of activin, follistatin, and inhibin in the regulation of Kiss-1 gene expression in hypothalamic

- cell models†. *Biol Reprod* 101: 405–415.
25. Tumurgan Z, Kanasaki H, Tumurbaatar T, Oride A, Okada H, *et al.* (2020) Roles of intracerebral activin, inhibin, and follistatin in the regulation of Kiss-1 gene expression: Studies using primary cultures of fetal rat neuronal cells. *Biochem Biophys Res* 23: 100785.
 26. Paxinos G, Watson C (2013) *The Rat Brain in Stereotaxic Coordinates* (7th). Academic Press, London, UK.
 27. Zhou L, Ren Y, Li D, Zhou W, Li C, *et al.* (2023) Timosaponin AIII attenuates precocious puberty in mice through downregulating the hypothalamic-pituitary-gonadal axis. *Acta Biochim Pol* 70: 183–190.
 28. Knight PG (1996) Roles of inhibins, activins, and follistatin in the female reproductive system. *Front Neuroendocrinol* 17: 476–509.
 29. Schmitt J, Hötten G, Jenkins NA, Gilbert DJ, Copeland NG, *et al.* (1996) Structure, chromosomal localization, and expression analysis of the mouse inhibin/activin beta C (*Inhbc*) gene. *Genomics* 32: 358–366.
 30. Reader KL, John-McHaffie S, Zellhuber-McMillan S, Jowett T, Mottershead DG, *et al.* (2022) Activin B and activin C have opposing effects on prostate cancer progression and cell growth. *Cancers (Basel)* 15: 147.
 31. Zhou X, Gu D, Hou G (2017) Erythromycin attenuates metalloprotease/anti-metalloprotease imbalance in cigarette smoke-induced emphysema in rats *via* the mitogen-activated protein kinase/nuclear factor- κ B activation pathway. *Mol Med Rep* 15: 2983–2990.
 32. Wong ML, Medrano JF (2005) Real-time PCR for mRNA quantitation. *Biotechniques* 39: 75–85.
 33. Bustin SA, Benes V, Nolan T, Pfaffl MW (2005) Quantitative real-time RT-PCR--a perspective. *J Mol Endocrinol* 34: 597–601.
 34. Treen AK, Luo V, Chalmers JA, Dalvi PS, Tran D, *et al.* (2016) Divergent regulation of ER and kiss genes by 17 β -estradiol in hypothalamic ARC *versus* AVPV models. *Mol Endocrinol* 30: 217–233.
 35. Lewis KA, Gray PC, Blount AL, MacConell LA, Wiater E, *et al.* (2000) Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. *Nature* 404: 411–414.
 36. Makanji Y, Zhu J, Mishra R, Holmquist C, Wong WP, *et al.* (2014) Inhibin at 90: from discovery to clinical application, a historical review. *Endocr Rev* 35: 747–794.
 37. Roberts V, Meunier H, Vaughan J, Rivier J, Rivier C, *et al.* (1989) Production and regulation of inhibin subunits in pituitary gonadotropes. *Endocrinology* 124: 552–554.
 38. Farnworth PG, Harrison CA, Leembruggen P, Chan KL, Stanton PG, *et al.* (2001) Inhibin binding sites and proteins in pituitary, gonadal, adrenal and bone cells. *Mol Cell Endocrinol* 180: 63–71.
 39. Besecke LM, Guendner MJ, Schneyer AL, Bauer-Dantoin AC, Jameson JL, *et al.* (1996) Gonadotropin-releasing hormone regulates follicle-stimulating hormone-beta gene expression through an activin/follistatin autocrine or paracrine loop. *Endocrinology* 137: 3667–3673.
 40. MacConell LA, Widger AE, Barth-Hall S, Roberts VJ (1998) Expression of activin and follistatin in the rat hypothalamus: anatomical association with gonadotropin-releasing hormone neurons and possible role of central activin in the regulation of luteinizing hormone release. *Endocrine* 9: 233–241.
 41. Hafez ES (1980) Male ad female inhibin. *Arch Androl* 5: 131–158.