Title

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Mutual Interactions between GnRH and Kisspeptin in GnRH- and Kiss-1-Expressing Immortalized Hypothalamic Cell Models

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Abstract

Background: Kisspeptin and gonadotropin-releasing hormone (GnRH) are central regulators of the hypothalamic-pituitary-gonadal axis and control female reproductive functions. Recently established mHypoA-50 and mHypoA-55 cells are immortalized hypothalamic neuronal cell models that originated from the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC) regions of the mouse hypothalamus, respectively.

Methods: mHypoA-50 or -55 cells were stimulated with kisspeptin-10 (KP10) and GnRH, after which the expression of kisspeptin and GnRH was determined. Primary cultures of fetal rat brain cells were also examined.

Results: mHypoA-50 and -55 cells expressed mRNA for Kiss-1 (which encodes kisspeptin) and GnRH as well as receptors for kisspeptin and GnRH. We found that Kiss-1 mRNA expression was significantly increased in mHypoA-50 AVPV cells by KP10 and GnRH stimulation. Kisspeptin protein expression was also increased by KP10 and GnRH stimulation in these cells. In contrast, GnRH expression was unchanged in mHypoA-50 AVPV cells by KP10 and GnRH stimulation. In mHypoA-55 ARC cells, kisspeptin expression was also significantly increased at the mRNA and protein levels by KP10 and GnRH stimulation; however, GnRH expression was also up-regulated by KP10 and GnRH stimulation in these cells. KP10 and estradiol (E2) both increased Kiss-1 gene expression in mHypoA-50 AVPV cells, but combined stimulation with KP10 and E2 did not potentiate their individual effects on Kiss-1 gene expression. On the other hand, E2 did not increase Kiss-1 gene expression in mHypoA-55 ARC cells, and the KP10-induced increase of Kiss-1 gene expression was inhibited in the presence of E2 in these cells. KP10 and GnRH significantly increased c-Fos protein expression in the mHypoA-50

AVPV and mHypoA-55 ARC cell lines. In primary cultures of fetal rat neuronal cells, KP10 significantly increased Kiss-1 gene expression, whereas GnRH significantly increased GnRH gene expression.

Conclusions: We found that kisspeptin and GnRH affected Kiss-1- and GnRH-expressing hypothalamic cells and modulated Kiss-1 and/or GnRH gene expression with a concomitant increase in c-Fos protein expression. A mutual- or self-regulatory system might be present in Kiss-1 and/or GnRH neurons in the hypothalamus.

Background

Kisspeptin and gonadotropin-releasing hormone (GnRH) in the hypothalamus play pivotal roles in the maintenance of female reproductive functions. For a long time, GnRH was believed to be positioned at the highest level of the hypothalamic-pituitary-gonadal (HPG) axis. However, after the discovery of inactivating mutations in the kisspeptin receptor (Kiss1r) of patients with idiopathic hypogonadotropic hypogonadism [1, 2], it gradually became clear that hypothalamic neurons that produce kisspeptin (encoded by the *KISS1* gene) control GnRH neurons in several mammalian species [3-5]. Therefore, at present, it is generally accepted that kisspeptin-expressing neurons (Kiss-1 neurons) play a pivotal role in maintaining the HPG axis by stimulating the release of GnRH. As GnRH neurons do not express estrogen receptor α (ERα) [6], the discovery of Kiss-1 neurons expressing ERα enabled the characterization of the mechanisms for sex steroid-induced feedback control of the HPG axis.

In rodents, Kiss-1 neurons are located in two different areas of the hypothalamus, namely, the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC), and control GnRH release by different mechanisms [3, 7]. Kiss-1 expression in the AVPV region is up-regulated by estradiol (E2), while it is repressed by E2 in the ARC region [8], suggesting that Kiss-1 neurons in the AVPV induce a surge in GnRH/luteinizing hormone release (positive feedback), whereas those in the ARC region are implicated in the E2-induced negative feedback regulation of gonadotropin secretion [9, 10]. Indeed, Kiss-1 neurons project to GnRH neurons in many different species. In mice, fibers from Kiss-1 neurons in the AVPV region connect directly to the cell bodies of GnRH neurons in the preoptic area, while neuronal fibers from Kiss-1 neurons in the ARC are in apposition to GnRH nerve endings that run through this region to the median eminence [11-13].

mHypoA-50 and mHypoA-55 cells are hypothalamic cell models that originated from Kiss-1 neurons in the AVPV and ARC regions of the adult mouse hypothalamus, respectively [14]. These cell lines share common characteristics with Kiss-1 neurons such as the expression of Kiss-1 and estrogen receptors (ERα, ERβ, and G-protein-coupled receptor 30). In contrast, there are differences between these two cell lines. For example, mHypoA-55 ARC cells express neurokinin B and dynorphin A, which are expressed by kisspeptin-neurokinin B-dynorphin A (KNDy) neurons in the ARC region, whereas mHypoA-50 AVPV cells do not express these peptides; instead, mHypoA-50 cells, but not mHypoA-55 cells, co-express tyrosine hydroxylase and Met-enkephalin [14].

GnRH neurons express Kiss1R [15], and kisspeptin increases their firing activity and GnRH release [16, 17]. Blockage of kisspeptin action by specific antibodies decreases GnRH activity [18]. Deletion of Kiss1R from GnRH neurons results in similar phenotypes as observed in Kiss1R knockout mice [19]. These observations indicate that kisspeptin is a crucial regulator of GnRH neurons through Kiss1R. In our series of experiments using the Kiss-1-expressing mHypoA-50 and -55 cell models to investigate the characteristics of Kiss-1 neurons, we found that they also express GnRH. Using these Kiss-1 and GnRH co-expressing neurons, we examined how Kiss-1 and GnRH were reciprocally regulated. In this study, we investigated the possible autocrine and paracrine regulation of kisspeptin and GnRH in these hypothalamic cell lines.

Materials and Methods

Materials

The following chemicals and reagents were obtained from the indicated sources: GIBCO fetal bovine serum (FBS; Invitrogen, Carlsbad, CA); GnRH, penicillin-streptomycin, and water-soluble E2 (Sigma-Aldrich Co., St. Louis, MO); and kisspeptin-10 (KP10) (AnaSpec, Fremont, CA).

Cell culture

mHypoA-50 and -55 cells were purchased from CEDARLANE (Ontario, Canada). The cells were plated in 35-mm tissue culture dishes and incubated with high-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Co.) 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 culture medium was changed to high-glucose DMEM containing 1% heat-inactivated FBS and 1% penicillin-streptomycin, and the cells were incubated without (control) or with 100 nM KP10 or 100 nM GnRH for 24 h.

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

Total RNA was extracted from stimulated cells 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 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, Kiss1R, GnRH, and GnRH receptor (GnRHR), after PCR

amplification using primers for Kiss-1 (forward: 5′-AGCTGCTGCTTCTCCTCTGT-3′ and reverse: 5′-GCATACCGCGATTCCTTTT-3′), Kiss1R (forward: 5′-CTGCCACAGACGTCACTTTC-3′ and reverse: 5′-ACATACCAGCGGTCCACACT-3′), GnRH (forward: 5′-ACTGTGTGTTTGGAAGGCTGC-3′ and reverse: 5′-TTCCAGAGCTCCTCGCAGATC-3′), and GnRHR (forward: 5′-CTAACAATGCGTCTCTTGA-3′ and reverse: 5′-TCCAGATAAGGTTAGAGTCG-3′), amplicons were electrophoresed in 1.5% agarose gels and visualized with ethidium bromide staining. Kiss-1 and GnRH mRNA levels were determined through quantitative RT-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 Fast Start Master Mix (Roche Diagnostics, Mannheim, Germany). Using specific primers for Kiss-1 and GnRH (as described above), 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 described previously [20]. PCR conditions were optimized to generate >95% PCR efficiency, and only those reactions with between 95% and 105% efficiency were included in subsequent analyses. Relative differences in cDNA concentrations between the baseline and experimental conditions were calculated using the comparative threshold cycle (Ct) method [21]. Briefly, for each sample, ΔCt was calculated to normalize expression to the internal control using the following equation: $\Delta Ct = \Delta Ct(gene) - Ct(GAPDH)$. To obtain 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 = $2^{\Delta\Delta Ct}$.

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 [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 × *g* for 10 min at 4°C. Protein concentration in the cell lysates was measured using the Bradford method. Denatured protein (30 μg) 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-Kiss-1 antibody (1:200 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX), anti-Kiss1R antibody (1:200 dilution; Abcam, Cambridge, UK), anti-GnRH antibody (1:500 dilution; ABclonal Technology, Inc., Boston, MA), anti-GnRHR antibody (1:200 dilution; Santa Cruz Biotechnology, Inc.), or anti-c-Fos antibody (1:200 dilution; Santa Cruz Biotechnology, Inc.) in Blotto overnight at 4°C and washed 3 times for 10 min per wash with Tris-buffered saline/1% Tween. A subsequent incubation with horseradish peroxidase (HRP)-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). After strip washing (Restore Buffer; Pierce Chemical Co., Rockford, IL), the membranes were reprobed with

171 an anti-β-actin antibody (1:5,000 dilution; Abcam) for 1 h at room temperature and
172 incubated with HRP-conjugated secondary antibodies before the procedure was
173 continued as described above. When the expression levels of kisspeptin and GnRH were
174 compared after KP10 and GnRH stimulation, the films were analyzed by densitometry,
175 and the intensity of kisspeptin or GnRH was normalized to that of β-actin to correct for
176 protein loading.
177
178 *Primary culture of neuronal cells from fetal rat brain*
179 Six to 8 fetal rat brains were obtained from fetuses from a female rat at 16–18
180 days of gestation under deep sodium pentobarbital anesthesia. Whole brains from fetal
181 rats were excised and minced before incubation in calcium- and magnesium-free Hank's
182 Balanced Salt Solution (CMF-HBSS) containing 10 mg/mL trypsin and 2 mg/mL
183 collagenase (Nitta Gelatin, Osaka, Japan) for 15 min at 37°C. The samples were incubated
184 in an identical solution containing 0.5 μg/mL DNase I (Boehringer-Mannheim,
185 Mannheim, Germany) for 5 min at 37°C. After incubation in CMF-HBSS containing 5
186 mM ethylenediaminetetraacetic acid (Wako Pure Chemicals, Osaka, Japan) for 5 min at
187 37°C, the samples were washed with CMF-HBSS. The dispersed cells were suspended in
188 CMF-HBSS using a pipette, passed through a 70-μm nylon mesh (Becton Dickinson
189 Labware, Franklin Lakes, NJ), and collected by centrifugation. The pellet was
190 resuspended and 2.0–3.0 × 10^5 cells were cultured on a 35-mm Petri dish in DMEM with
191 10% FBS and 1% penicillin-streptomycin until use. This protocol was approved by the
192 committee of the Experimental Animal Center for Integrated Research in Shimane
193 University.
194

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. From four sets of data, we calculated the mean ± standard error. Averages from independent experiments were statistically analyzed. Data are expressed as the mean ± standard error of the mean (SEM) values. Statistical analysis was performed using one-way or two-way analysis of variance with Bonferroni's *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Kiss-1, GnRH, Kiss1R, and GnRHR expression in the mHypoA-50 and -55 hypothalamic cell lines

RT-PCR analysis showed that the mHypoA-50 and -55 cell lines expressed mRNA for kisspeptin and GnRH as well as their receptors (Fig. 1A). Kisspeptin and GnRH as well as their receptors were detected at the protein level in these cell lines (Fig. 1B). These genes and proteins were also detected in the GnRH-producing GT1-7 cell line and primary cultured neuronal cells from fetal rat brain.

Effect of KP10 and GnRH on kisspeptin and GnRH expression in the mHypoA-50 AVPV cell line

mHypoA-50 AVPV cells were stimulated with 100 nM KP10 or 100 nM GnRH and kisspeptin and GnRH expression was examined. KP10 stimulation significantly increased Kiss-1 mRNA expression by 3.25 ± 0.39-fold in mHypoA-50 cells (Fig. 2A). Kiss-1 mRNA was also significantly increased (3.66 ± 1.04-fold) in these cells by GnRH stimulation (Fig. 2A). Although KP10 stimulation did not induce a significant increase in kisspeptin expression, GnRH stimulation significantly increased kisspeptin protein levels in mHypoA-50 cells (Fig. 2C, D). In contrast, GnRH stimulation did not change the expression of kisspeptin or GnRH at the mRNA and protein levels (Fig. 2B, C, E).

Effect of KP10 and GnRH on kisspeptin and GnRH expression in the mHypoA-55 ARC cell line

mHypoA-55 ARC cells were stimulated with 100 nM KP10 or 100 nM GnRH and kisspeptin and GnRH expression was examined. KP10 and GnRH stimulation

significantly increased Kiss-1 gene expression by 2.06 ± 0.50-fold and 1.97 ± 0.14-fold, respectively (Fig. 3A). Kisspeptin protein expression was also increased by KP10 (1.83 ± 0.05-fold) and GnRH (1.68 ± 0.10-fold) stimulation compared with non-stimulated control cells (Fig. 3C, D). In mHypoA-55 ARC cells, GnRH mRNA expression was significantly increased by KP10 (2.08 ± 0.16-fold) and GnRH (1.45 ± 0.14-fold) stimulation (Fig. 3B). Similarly, GnRH protein expression was significantly up-regulated in mHypoA-55 cells by KP10 and GnRH stimulation (Fig. 3C, E).

Effect of E2 on KP10-induced Kiss-1 gene expression in the mHypoA-50 and -55 hypothalamic cell lines

Next, we examined the effect of E2 on Kiss-1 gene expression in the AVPV and ARC cell models. As shown above, KP10 stimulation significantly increased Kiss-1 gene expression in the mHypoA-50 AVPV and mHypoA-55 ARC cell models. However, the effect of E2 on Kiss-1 gene expression was distinct in each cell line. When the cells were treated with 100 nM E2 for 24 h, Kiss-1 gene expression was significantly increased in mHypoA-50 AVPV cells by 2.46 ± 0.27-fold compared to non-stimulated control cells (Fig. 4A). On the other hand, E2 failed to modulate Kiss-1 gene expression in mHypoA-55 ARC cells (Fig. 4B). Combined stimulation with KP10 and E2 did not potentiate their individual effects on Kiss-1 gene expression in mHypoA-50 AVPV cells, while the KP10-induced increase of Kiss-1 gene expression in mHypoA-55 ARC cells was inhibited in the presence of E2 (Fig. 4A and B).

Effect of KP10 and GnRH on c-Fos expression in the mHypoA-50 and -55 hypothalamic cell lines

c-Fos protein is a marker of neuronal activity following neuronal stimulation [22]. In mHypoA-50 AVPV and mHypoA-55 ARC cells, KP10 (100 nM) and GnRH (100 nM) stimulation significantly increased c-Fos protein expression (Fig. 5A, B).

Effect of KP10 and GnRH on Kiss-1 and GnRH mRNA expression in neuronal cultures from fetal rat brain

Finally, we examined the effects of KP10 or GnRH stimulation in primary cultures of fetal rat brain that contained Kiss-1 and GnRH neurons. Exogenous KP10 (100 nM) stimulation increased Kiss-1 mRNA expression by 2.20 ± 0.39-fold compared to non-stimulated control cells in these primary cultures. Although GnRH increased Kiss-1 gene expression by 1.6 ± 0.19-fold, it was not a statistically significant increase compared to control cells (Fig. 6A). Conversely, GnRH stimulation significantly increased GnRH mRNA expression by 1.99 ± 0.31-fold compared to non-stimulated cells. KP10 failed to induce a significant increase in GnRH mRNA expression in these cells (Fig. 6B).

Discussion

Previous *in vivo* studies using animals or *in vitro* studies using hypothalamic tissues have proven that kisspeptin from Kiss-1 neurons regulates the release of GnRH from GnRH neurons via Kiss1R. To examine the cellular mechanisms involved in Kiss-1 gene expression, we have used the murine-derived hypothalamic mHypoA-50 and -55 cell models, which originated from the AVPV and ARC regions of the hypothalamus, respectively. In our series of studies using these hypothalamic cell models, we found that they also express GnRH. As mHypoA-50 and -55 cells express Kiss1R and GnRHR, we hypothesized that mutual regulation exists between kisspeptin and GnRH in these cells.

In the present study, we showed that interactions exist between kisspeptin and GnRH signaling in these Kiss-1- and GnRH-expressing cells. KP10 and GnRH increased Kiss-1 expression in mHypoA-50 AVPV cells. Similarly, Kiss-1 mRNA expression was increased in mHypoA-55 ARC cells by KP10 and GnRH stimulation. As for GnRH expression, KP10 and GnRH increased GnRH mRNA expression in mHypoA-55 AVPV cells, but not in mHypoA-50 AVPV cells. Furthermore, we observed that KP10 and GnRH stimulation increased c-Fos protein expression in each cell model, indicating that neuronal activity was activated in these cells by KP10 and/or GnRH. Considering these observations, we suspected that mutual interactions exist between kisspeptin and GnRH in these Kiss-1- and GnRH-expressing neurons.

However, we first need to clarify the nature of the cell models used in this study. A number of hypothalamic neuronal cell lines from embryonic mice or rats have been established using the SV-40 large T-antigen with a lentiviral vector [14, 23, 24]. As the hypothalamus is comprised of a complex network of neurons, there are distinct neuronal phenotypes within a complex array of neurons expressing specific complements of

293 neuropeptides, neurotransmitters, and receptors [25]. mHypoA-50 and -55 cells were
294 established from the AVPV and ARC regions, respectively, of Kiss-1-GFP transgenic
295 adult mice using fluorescence-activated cell sorting techniques [14]. Among several cell
296 lines that expressed Kiss-1 mRNA, these two were chosen as models of Kiss-1 neurons
297 because they expressed high levels of Kiss-1 mRNA as well as ERα, ERβ, and G-protein-
298 coupled receptor 30. Subsequently, mHypoA-55 cells were found to express
299 neuropeptides, such as dynorphin A, neurokinin B, and substance P, which are also
300 expressed by KNDy neurons *in vivo* [26, 27]. In contrast, mHypoA-50 cells are devoid of
301 these peptides; instead, they co-express Met-enkephalin and thymidine hydroxylase, both
302 of which are expressed in Kiss-1 neurons in the AVPV region of the hypothalamus of
303 mice *in vivo* [28, 29]. Thus, although these cells have similar characteristics as Kiss-1
304 neurons *in vivo*, they are still artificially produced hypothalamic cell models, which were
305 immortalized from primary cell cultures of microdissected hypothalamus. As mHypoA-
306 55 ARC cells and mHypoA-50 AVPV cells express Kiss-1 and GnRH as well as their
307 receptors, it is plausible that similar neurons exist in the hypothalamus *in vivo*. Indeed,
308 Kiss-1 mRNA and kisspeptin are expressed not only in the AVPV and ARC regions of the
309 hypothalamus but also throughout the brain, including the dorsomedial nucleus, posterior
310 hypothalamus, and amygdala [5, 7, 30]. Kiss-1 mRNA is also detected in the preoptic area
311 of the hypothalamus, where GnRH neurons are present [31]. Similarly, although Kiss1R
312 is predominantly expressed by GnRH neurons in the preoptic area, its expression is
313 detected broadly in non-GnRH neurons in various brain regions, including the ARC [32,
314 33]. Indeed, another hypothalamic cell line, mHypo36/1, expresses Kiss-1 and Kiss1R
315 [24]. GnRH immunoreactive cells are also detected outside of the preoptic area, including
316 the ARC [34]. Furthermore, GnRHR is expressed ubiquitously in organs including whole

317 brain regions in humans and primates [35, 36]. These previous observations suggest that
318 neurons expressing Kiss-1 and GnRH together with Kiss1R and GnRHR are present in
319 the hypothalamus, or similar cells might exist temporarily during the development of
320 Kiss-1 or GnRH neurons. Regarding the mHypoA-55 and -50 Kiss-1-expressing cell lines,
321 the initial report by Treen et al. did not mention GnRH, GnRHR, and Kiss1R expression
322 in these cells [14]. However, these cells also express other peptides such as neurotensin,
323 corticotropin-releasing hormone, pituitary adenylate cyclase-activating polypeptide, RF
324 amide-related peptide 3, and inhibin subunits [37-40]. These observations suggest that
325 neurons might be able to produce a variety of peptides and receptors or they are totipotent.
326 GT1-7 cells are used widely as a model for GnRH neurons, and were
327 immortalized by genetically targeted tumorigenesis using the promoter region of GnRH
328 to express the SV40 T-antigen oncogene [41]. The GT1-7 cell line was classically known
329 as a GnRH-secreting cell model, but it also produces and secretes kisspeptin [42]. In this
330 study, we confirmed that our GT1-7 cell line expresses the Kiss-1 gene in addition to
331 GnRH. In our series of previous experiments using GT1-7 cells, exogenous KP10
332 stimulation failed to increase GnRH expression, while it increased GnRHR levels [43].
333 Although GT1-7 cells are defined as a model for GnRH neurons, it is obscure whether
334 these cells possess the original characteristics of GnRH neurons *in vivo*. Conversely,
335 GnRH expression in our mHypoA-55 cells was increased by KP10. Although it is still
336 unknown which hypothalamic cell models are suitable to study the cellular mechanisms
337 underlying the regulation of Kiss-1 or GnRH, GnRH expression in mHypoA-55 cells
338 responded to kisspeptin, as observed *in vivo*. Thus, we used these cells as a model for
339 GnRH- or Kiss-1-producing neuronal cells, which have many of the original
340 characteristics of putative hypothalamic neurons.

Regarding the Kiss-1 gene, KP10 increased its expression in mHypoA-50 AVPV cells and mHypoA-55 ARC cells, suggesting that kisspeptin could be controlled in an autocrine/paracrine manner by kisspeptin itself. Furthermore, Kiss-1 gene expression was also upregulated by GnRH in both of these cell lines, suggesting that GnRH could increase Kiss-1 levels in hypothalamic neurons. Given that Kiss-1 neurons in the AVPV region of the hypothalamus are implicated in E2-induced positive feedback, this phenomenon could increase GnRH release via kisspeptin release. However, it is still unknown why GnRH increases Kiss-1 levels in Kiss-1 neurons of the ARC region, which is involved in E2-induced negative feedback control. GnRH gene expression in mHypoA-50 AVPV cells was unchanged by KP10 and GnRH stimulation, but was increased by both of these peptides in mHypoA-55 ARC cells. These observations suggest that Kiss-1- and GnRH-expressing cells originate from different areas of the hypothalamus and have distinct characteristics, as reported previously. In addition, we found that GnRH gene expression in the ARC region of the hypothalamus was more sensitive to kisspeptin and GnRH stimulation and was increased by these peptides. Although GnRH is under the control of kisspeptin, which is released by upstream Kiss-1 neurons, GnRH might also be regulated in an autocrine/paracrine manner in GnRH-expressing neurons in this area.

In this study, we confirmed that two different hypothalamic cell models, mHypoA-50 and -55 cells, possess distinct characteristics with respect to their response to E2. mHypoA-50 AVPV cells responded to E2 and increased Kiss-1 expression. In contrast, Kiss-1 gene expression was not modulated by E2 in mHypoA-55 ARC cells. These observations are comparable to those observed in previous reports [14, 40]. In addition to E2, KP10 increased Kiss-1 gene expression in mHypoA-50 AVPV cells, indicating that kisspeptin itself is also involved in a positive feedback mechanism, which

365 is controlled by Kiss-1 neurons in the AVPV region. As combined treatment of mHypoA-
366 50 AVPV cells with E2 and KP10 did not potentiate their individual positive effects on
367 Kiss-1 gene expression, E2 and kisspeptin might not cooperate. Conversely, although 4-
368 h treatment with E2 reportedly represses Kiss-1 expression in mHypoA-55 ARC cells [14,
369 40], 24-h treatment with E2 had no effect on its expression. However, the induction of
370 Kiss-1 expression by KP10 stimulation was significantly repressed in the presence of E2.
371 These observations indicate that in addition to the direct inhibitory effect of E2 on Kiss-
372 1 gene expression in the ARC region of the hypothalamus, E2 also has an inhibitory effect
373 on the ability of kisspeptin to induce Kiss-1 gene expression.

374 To confirm further the induction of Kiss-1 and GnRH gene expression by
375 kisspeptin or GnRH in the brain, we used primary cultures of fetal rat brain cells. We
376 observed that KP10 increased Kiss-1 mRNA expression, whereas GnRH increased GnRH
377 mRNA expression in these cultures. As these primary cultures contain a variety of
378 neuronal phenotypes, it is plausible to consider that autoregulatory systems exist in Kiss-
379 1- or GnRH-expressing neurons. It has been reported that kisspeptin increases GnRH
380 secretion in the GT1-7 cell line, but GnRH decreases kisspeptin secretion in these cells
381 [42]. Kisspeptin also stimulates GnRHR expression in GT1-7 cells [43]. These reports
382 suggest mutual regulation between kisspeptin and GnRH. However, it is still not known
383 whether Kiss-1 neurons and GnRH neurons mutually interact via synaptic connections.
384 There is a possibility that the phenomena observed in this study occur only in neurons
385 that express Kiss-1 and GnRH mRNAs. Furthermore, it remains unclear whether these
386 phenomena are limited to rodent models. Investigations using other animal models are
387 required to determine the detailed regulatory mechanisms of kisspeptin and GnRH, both
388 of which are central regulators of the HPG axis.

Precise control of the HPG axis is essential for maintaining fertility in all animals. Understanding the physiology of Kiss-1/GnRH neurons may enable us to take the next step toward developing a means by which we can manipulate the HPG axis. This knowledge would be applicable to the treatment of hypogonadotropic hypogonadism, delayed puberty, and hypothalamic amenorrhea as well as the development of a better approach for inducing ovarian stimulation.

Conclusions

In this study, we found that kisspeptin and GnRH affect Kiss-1- and GnRH-expressing hypothalamic cells and modulate Kiss-1 and/or GnRH gene expression with a concomitant increase in c-Fos protein expression. An autocrine or mutual regulation system might exist in hypothalamic Kiss-1- or GnRH-expressing neurons.

Abbreviations

ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; GnRH, gonadotropin-releasing hormone; GnRHR, gonadotropin-releasing hormone receptor; Kiss1R, kisspeptin receptor; KNDy, kisspeptin-neurokinin B-dynorphin A; KP10, kisspeptin-10

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Authors' contributions

HK and SA conceived and designed the experiments. HK, TT, ZT, AO, and HO performed

the experiments. HK wrote the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

The study protocol was approved by the committee of the Experimental Animal Center for Integrated Research, Shimane University. Consent to participate is not applicable in this study.

Availability of supporting data

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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Figure Legends

Fig. 1

Kiss-1, Kiss1R, GnRH, and GnRHR expression in mHypoA-50 and -55 hypothalamic cell lines. (A) Total RNA was prepared and RT-PCR was carried out for 35 cycles using primers specific for Kiss-1, Kiss1R, GnRH, and GnRHR. PCR products were resolved in 1.5% agarose gels and visualized with ethidium bromide staining. (B) Cell lysates (30 μg protein) from mHypoA-50 and -55 cells were analyzed by SDS-PAGE followed by immunoblotting and incubation with antibodies against Kiss-1, Kiss1R, GnRH, and GnRHR. The bands were visualized using HRP-conjugated secondary antibodies.

Fig. 2

Effect of KP10 and GnRH on kisspeptin and GnRH expression in mHypoA-50 AVPV cells. mHypoA-50 cells were treated with 100 nM KP10 or 100 nM GnRH for 24 h. Then, Kiss-1 (A) and GnRH (B) mRNA levels were measured by quantitative RT-PCR after mRNA extraction and reverse transcription. Samples for each experimental group were run in duplicate and normalized to GAPDH mRNA levels as a housekeeping gene. After stimulating the cells with KP10 or GnRH for 24 h, kisspeptin (C, D) and GnRH (C, E) protein levels were determined by western blotting and quantified, as described in the Materials and Methods. Results are expressed as fold stimulation over the unstimulated group/control. Values are means ± SEM of fold stimulation from independent experiments. *$P < 0.05$ vs. control.

Fig. 3

587 Effect of KP10 and GnRH on kisspeptin and GnRH expression in mHypoA-55 ARC cells.
588 mHypoA-55 cells were treated with 100 nM KP10 or 100 nM GnRH for 24 h. Then, Kiss-
589 1 (A) and GnRH (B) mRNA levels were measured by quantitative RT-PCR after mRNA
590 extraction and reverse transcription. Samples for each experimental group were run in
591 duplicate and normalized to GAPDH mRNA levels as a housekeeping gene. After
592 stimulating the cells with KP10 or GnRH for 24 h, kisspeptin (C, D) and GnRH (C, E)
593 protein levels were determined by western blotting and quantified, as described in the
594 Materials and Methods. Results are expressed as fold stimulation over the unstimulated
595 group/control. Values are means ± SEM of fold stimulation from independent
596 experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control.
597
598 **Fig. 4**
599 Effect of E2 on Kiss-1 mRNA expression in mHypoA-50 AVPV and -55 ARC cells.
600 mHypoA-50 (A) and -55 cells (B) were treated with 100 nM KP10 in the presence or
601 absence of 100 nM E2 for 24 h. Then, Kiss-1 mRNA levels were measured by quantitative
602 RT-PCR after mRNA extraction and reverse transcription. Samples for each experimental
603 group were run in duplicate and normalized to GAPDH mRNA levels as a housekeeping
604 gene. Results are expressed as fold stimulation over the unstimulated group/control.
605 Values are means ± SEM of fold stimulation from independent experiments. $^{**}P < 0.01$,
606 $^{*}P < 0.05$ vs. control. The difference between KP10 and KP10+E2 in mHypoA-55 cells
607 were statistically significant ($P < 0.05$).
608
609 **Fig. 5**
610 Effect of KP10 and GnRH on c-Fos protein expression in mHypoA-50 and -55 cells. The

611 cells were stimulated with 100 nM KP10 or 100 nM GnRH for 24 h, after which the
612 protein levels of c-Fos were analyzed in mHypoA-50 (A) and mHypoA-55 (B) cells by
613 western blotting.
614
615 **Fig. 6**
616 Effect of KP10 and GnRH on Kiss-1 and GnRH mRNA expression in primary cultures of
617 fetal rat brain cells. Neuronal cells from fetal rat brain were treated with 100 nM KP10 or
618 100 nM GnRH for 24 h. Then, Kiss-1 (A) and GnRH (B) mRNA levels were measured
619 by quantitative RT-PCR after mRNA extraction and reverse transcription. Samples for
620 each experimental group were run in duplicate and normalized to GAPDH mRNA levels
621 as a housekeeping gene. Results are expressed as fold stimulation over the unstimulated
622 group/control. Values are means ± SEM of fold stimulation from independent
623 experiments. *$P < 0.05$ vs. control.
624

Fig. 1

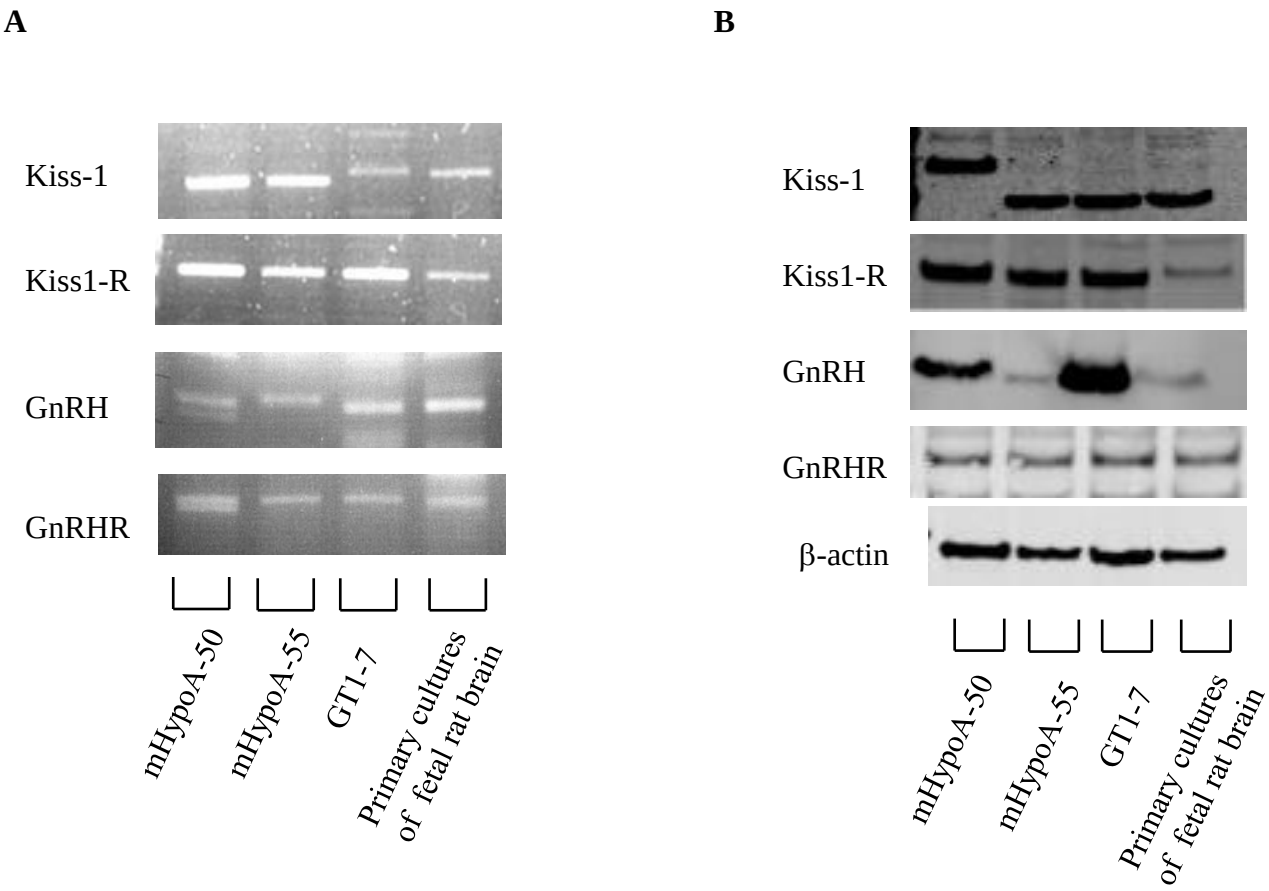

A
Kiss-1
Kiss1-R
GnRH
GnRHR
mHypoA-50
mHypoA-55
GT1-7
Primary cultures of fetal rat brain
B
Kiss-1
Kiss1-R
GnRH
GnRHR
β-actin
mHypoA-50
mHypoA-55
GT1-7
Primary cultures of fetal rat brain

Fig. 2

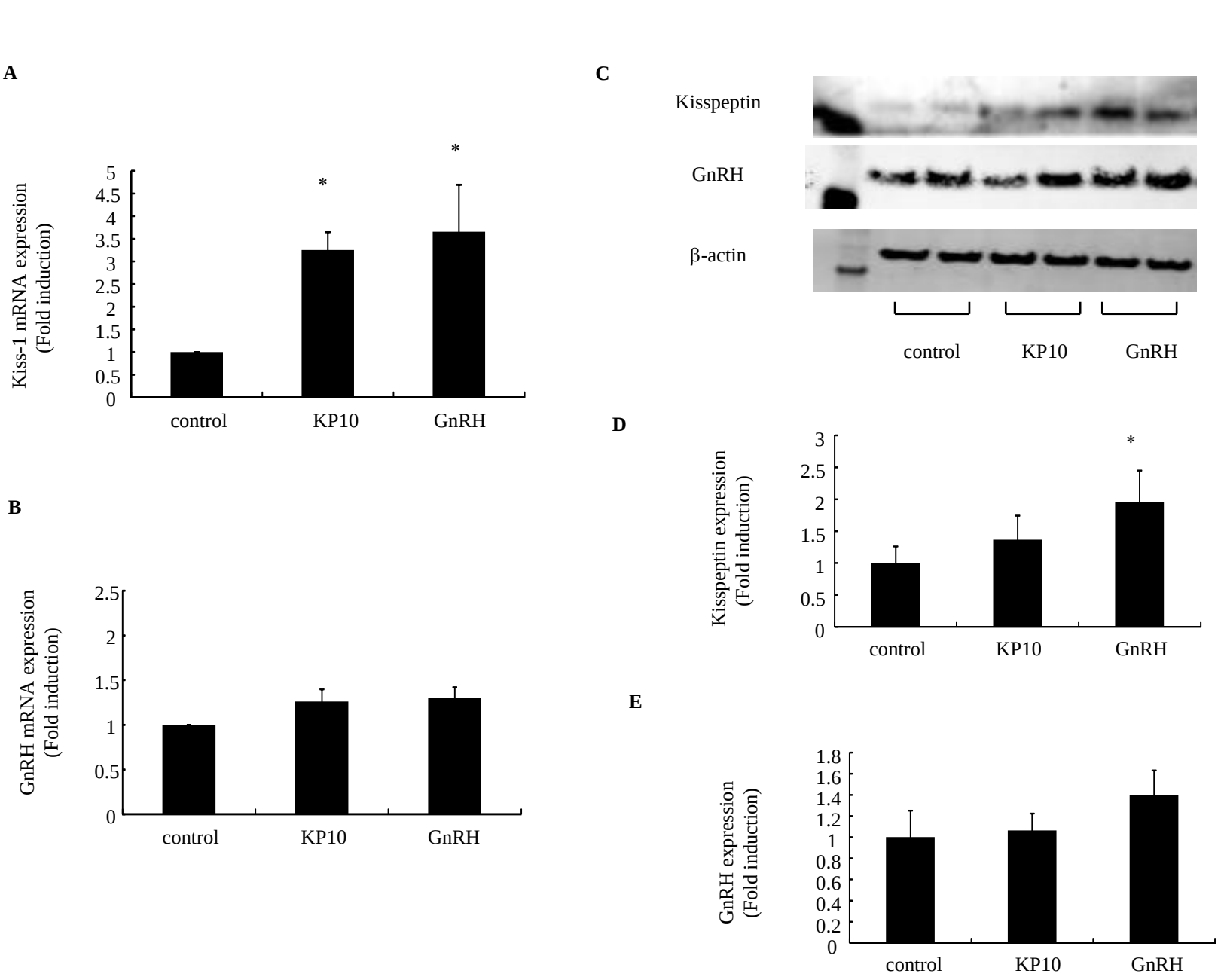
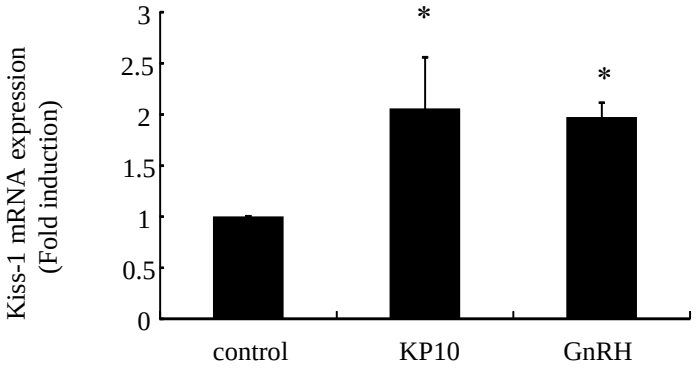

mHypoA-50
A
Kiss-1 mRNA expression
(Fold induction)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
control
KP10
GnRH
B
GnRH mRNA expression
(Fold induction)
2.5
2
1.5
1
0.5
0
control
KP10
GnRH
C
Kisspeptin
GnRH
β-actin
control
KP10
GnRH
D
Kisspeptin expression
(Fold induction)
3
2.5
2
1.5
1
0.5
0
*
control
KP10
GnRH
E
GnRH expression
(Fold induction)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
control
KP10
GnRH

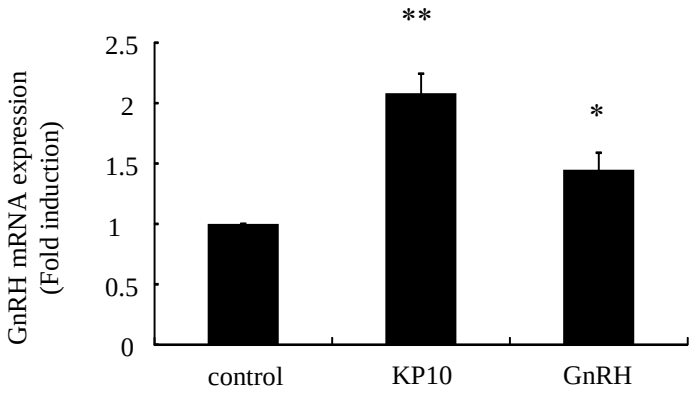
Fig. 3

mHypoA-55

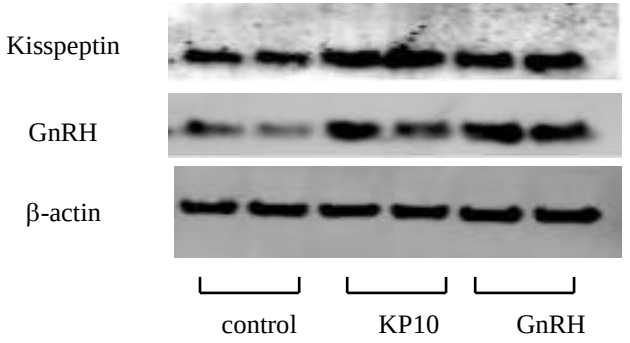
A



B



C



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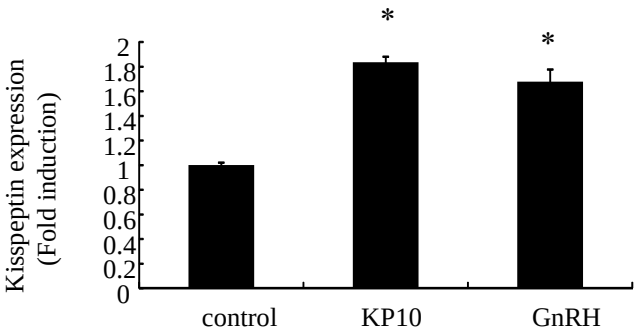

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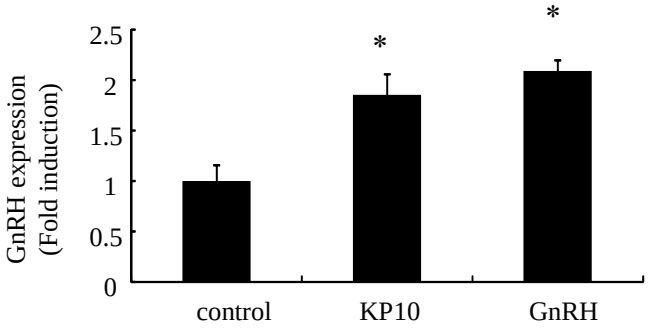


Fig. 4

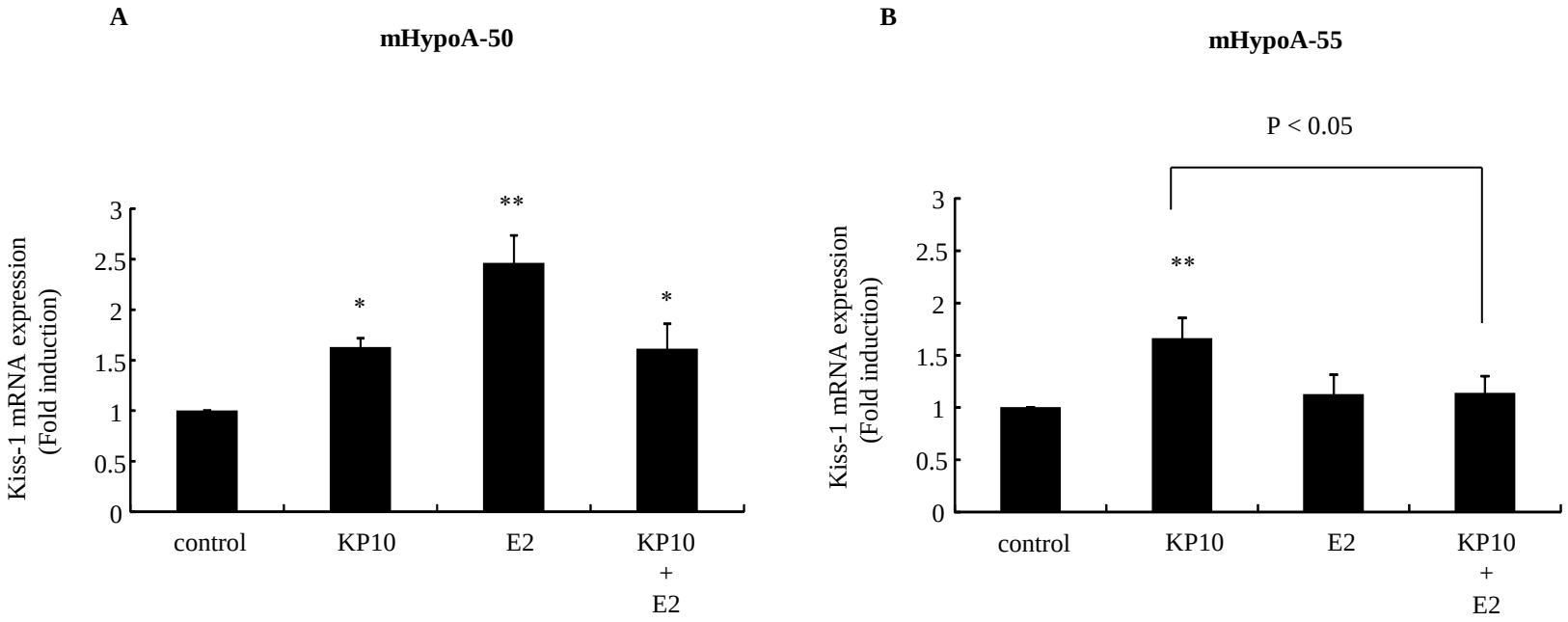

A
mHypoA-50
Kiss-1 mRNA expression
(Fold induction)
3
2.5
2
1.5
1
0.5
0
*
**
*
control
KP10
E2
KP10
+
E2
B
mHypoA-55
P < 0.05
Kiss-1 mRNA expression
(Fold induction)
3
2.5
2
1.5
1
0.5
0
**
control
KP10
E2
KP10
+
E2

Fig. 5

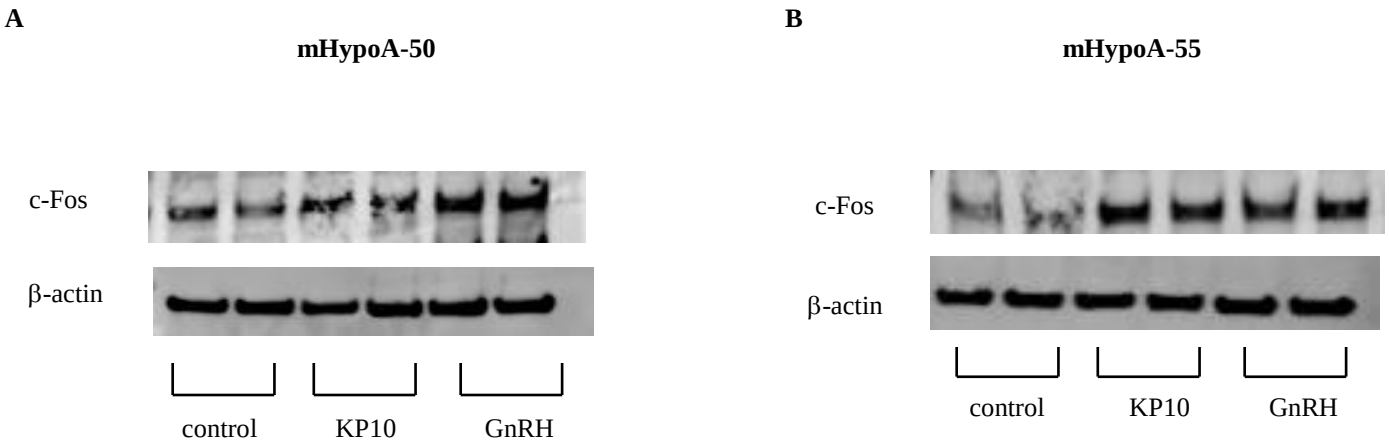
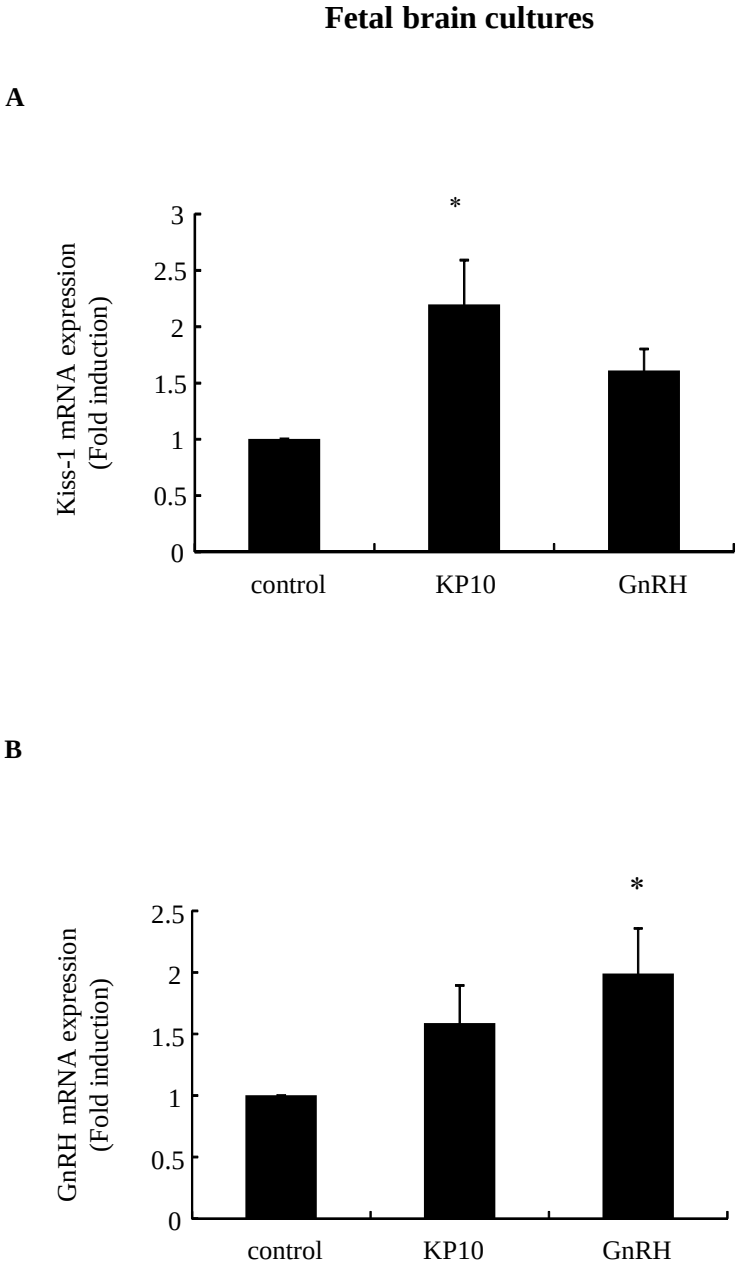

A
mHypoA-50
c-Fos
β-actin
control
KP10
GnRH
B
mHypoA-55
c-Fos
β-actin
control
KP10
GnRH

Fig. 6


Fetal brain cultures
A
Kiss-1 mRNA expression
(Fold induction)
3
2.5
2
1.5
1
0.5
0
*
control
KP10
GnRH
B
GnRH mRNA expression
(Fold induction)
2.5
2
1.5
1
0.5
0
*
control
KP10
GnRH