Regulation of GNRH production by estrogen and bone morphogenetic proteins in GT1-7 hypothalamic cells

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Abstract

Recent studies have shown that bone morphogenetic proteins (BMPs) are important regulators in the pituitary–gonadal endocrine axis. We here investigated the effects of BMPs on GNRH production controlled by estrogen using murine GT1-7 hypothalamic neuron cells. GT1-7 cells expressed estrogen receptor α (ERα; ESR1 as listed in MGI Database), ERβ (ESR2 as listed in MGI Database), BMP receptors, SMADs, and a binding protein follistatin. Treatment with BMP2 and BMP4 had no effect on *Gnrh* mRNA expression; however, BMP6 and BMP7 significantly increased *Gnrh* mRNA expression as well as GnRH production by GT1-7 cells. Notably, the reduction of *Gnrh* expression caused by estradiol (E_2) was restored by cotreatment with BMP2 and BMP4, whereas it was not affected by BMP6 or BMP7. E_2 activated extracellular signal-regulated kinase (ERK) 1/2 and stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK) signaling but did not activate p38-mitogen-activated protein kinase (MAPK) signaling in GT1-7 cells. Inhibition of ERK1/ERK2 reversed the inhibitory effect of estrogen on *Gnrh* expression, whereas SAPK/JNK inhibition did not affect the E_2 actions. Expression levels of *Erα* and *Erβ* were reduced by BMP2 and BMP4, but were increased by BMP6 and BMP7. Treatment with an ER antagonist inhibited the E_2 effects on *Gnrh* suppression including reduction of E_2-induced ERK phosphorylation, suggesting the involvement of genomic ER actions in *Gnrh* suppression. BMP2 and BMP4 also suppressed estrogen-induced phosphorylation of ERK1/ERK2 and SAPK/JNK signaling, suggesting that BMP2 and BMP4 downregulate estrogen effects by attenuating ER–MAPK signaling. Considering that BMP6 and BMP7 increased the expression of α1E-subunit of R-type calcium channel (*Cacna1e*), which is critical for GNRH secretion, it is possible that BMP6 and BMP7 directly stimulate GNRH release by GT1-7 cells. Collectively, a newly uncovered interaction of BMPs and ER may be involved in controlling hypothalamic GNRH production and secretion via an autocrine/paracrine mechanism.

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Introduction

Reproduction in mammals is controlled by the coordination between episodic release of GNRH and GNRH-dependent secretion of pituitary gonadotropins. GNRH is a highly conserved decapeptide, which is secreted by a scattered group of cells located in the hypothalamus. The pattern of GNRH secretion is regulated by the intrinsic oscillatory activity of GNRH neurons and the integration of presynaptic inputs of various neurotransmitters. GNRH synthesis and release are tightly regulated by gonadal steroids, which maintain control through a negative feedback system (Petersen *et al.* 2003). In females, estrogens act directly or indirectly on GNRH neuronal network to modulate the final output of GNRH into the median eminence (Herbison 1998, Herbison & Pape 2001). Estrogen receptors (ER) belong to the superfamily of nuclear receptors and are classified as class I ligand-activated transcription factors. There are two identified subtypes of ERα and ERβ (ESR1 and ESR2 as listed in MGI Database), which are encoded by separate genes and differ in structure, function, and anatomical distribution.

The characterization of specific estrogen effects and identification of ER-dependent signaling pathways have been difficult in GNRH neurons. In this respect, immortalized GNRH-producing GT1-7 cells have proven to be a valuable tool to study the biology of GNRH neurons (Liposits *et al.* 1991, Wetsel *et al.* 1991). Although earlier studies indicated that GNRH neurons generally lack steroid hormone receptors, recent studies have provided evidence that GT1-7 cells express functional ER, suggesting that estrogen regulates GNRH production and secretion directly in GNRH neuron in the reproductive system composed of hypothalamo-pituitary–ovarian (HPO) axis.

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Bone morphogenetic proteins (BMPs), which belong to the transforming growth factor-β (TGF-β or TGFB1 as listed in MGI Database) superfamily, were originally identified as the active components in bone extracts capable of inducing bone formation at ectopic sites. Recent studies have shown that BMPs are crucial molecules in normal folliculogenesis by regulating gonadotropin-induced steroidogenesis and mitosis in ovarian granulosa cells (Otsuka *et al.* 2000, Otsuka & Shimasaki 2002*a*, Shimasaki *et al.* 2004). The major regulatory process by BMPs in ovarian steroidogenesis is the control of FSH receptor signaling (Otsuka *et al.* 2001*a*,*b*), leading to normal follicular development in the ovary with prevention of immature ovulation. Furthermore, the pituitary BMP system acts as a regulator not only for pituitary differentiation but also for the transformation of differentiated pituitary cells (Otsuka & Shimasaki 2002*b*, Takeda *et al.* 2003, 2007, Miyoshi *et al.* 2008). It is of note that BMPs activate FSH production by the pituitary gonadotrope (Huang *et al.* 2001, Otsuka & Shimasaki 2002*b*, Nicol *et al.* 2008). Functional communication between the ovary and central nervous system ensures that the neural signal for ovulation occurs when ovarian follicles are fully matured. Hence, BMPs play crucial roles in female reproduction not only by regulating ovarian steroidogenesis and mitogenesis but also by activating pituitary gonadotropin secretion in an autocrine/paracrine manner.

However, the effects of BMPs on hypothalamic GNRH production and secretion have yet to be elucidated. In the present study, we here investigated the effects of BMPs on GNRH production controlled by estrogen using murine GT1-7 hypothalamic neuron cells. A novel interaction between BMPs and ER, which is involved in controlling hypothalamic GNRH production and secretion, was here uncovered.

Materials and Methods

Reagents and supplies

DMEM, dimethyl sulfoxide, penicillin–streptomycin solution, 17β-estradiol (E_2), BSA-conjugated E_2 (E_2-BSA), ICI 182 780 (also called fulvestrant), and recombinant human activin A were purchased from Sigma–Aldrich Co. Ltd. Recombinant human BMP-2, -4, -6, and -7 were purchased from R&D Systems Inc. (Minneapolis, MN, USA), recombinant human platelet-derived growth factor (PDGF)-BB was purchased from PeproTech (London, UK), and U0126 and SB203580 were purchased from Promega Corp. SP600125 was purchased from Biomol Lab. Inc (Plymouth Meeting, PA, USA). Mouse ovary total RNA was purchased from Ambion (Austin, TX, USA). For the experiments using E_2-BSA, the preparation of E_2-BSA free of estradiol was performed as previously reported (Taguchi *et al.* 2004, Takahashi *et al.* 2008): 4 ml E_2-BSA (1·25 mM in estrogen dissolved in 50 mM Tris–HCl (pH 8·5)) was added to a Amicon Ultra centrifugal filter unit with a MW cut off of 5000 (Millipore, Bedford, MA, USA) and centrifuged at 4000 ***g*** until 50 ml retentate remained. The retentate was washed with Tris buffer, and final volume was adjusted to 5 ml (1 mM).

GT1-7 cell culture

GT1-7 cells were kindly provided by Dr Pamela L Mellon, University of California, San Diego, CA, USA. GT1-7 cells were maintained in DMEM supplemented with 10% FCS, penicillin, and streptomycin (Sigma–Aldrich Corp.) at 37 °C in 5% CO_2 humidified atmosphere. The culture medium was changed twice a week, and cultures were passaged at ∼80% confluence. Changes in cell morphology and growing conditions were carefully monitored under an inverted microscope.

RNA extraction, RT-PCR, and quantitative real-time PCR analysis

GT1-7 cells (2×10^5 viable cells/ml) were precultured in serum-free DMEM, and cells were treated with indicated concentrations of BMPs in combination with E_2 and various chemical inhibitors including U0126, SB203580, SP600125, and ICI 182 780. After 24-h culture, the medium was removed, and total cellular RNAs were extracted using TRIzol (Invitrogen Corp.), subsequently quantified by measuring absorbance at 260 nm and stored at −80 °C until assay. The expression of BMP receptors (*Bmpr*s), *Smad*s, *Er*s, and *Gnrh* was detected by RT-PCR analysis. The extracted RNA (1 μg) was subjected to a RT reaction using First-Strand cDNA Synthesis System (Invitrogen Corp.) with random hexamer (2 ng/μl), reverse transcriptase (200 U), and dNTP (0·5 mM) at 42 °C for 50 min, 70 °C for 10 min. Subsequently, hot-start PCR was performed using $MgCl_2$ (1·5 mM), dNTP (0·2 mM), and Taq DNA polymerase (2·5 U) (Invitrogen Corp). Oligonucleotides used for PCR were custom ordered from Invitrogen Corp. PCR primer pairs were selected from different exons of the corresponding genes to discriminate PCR products that might arise from possible chromosome DNA contaminants. The primer pairs for mouse BMP receptors, Smads, and a housekeeping gene, ribosomal protein-L19 (*Rpl19*), were selected as reported (Kano *et al.* 2005, Takeda *et al.* 2007, Miyoshi *et al.* 2008). For *Er*s, *Gnrh*, and calcium channel α1E-subunit (*Cacna1e*) genes, the following sequences were used: *Erα*, 1523–1543 and 1737–1757 from GenBank accession number NM_007956; *Erβ*, 701–721 and 1004–1024 from NM_010157; *Gnrh*, 90–110 and 316–336 from BC116897; and calcium channel α1E, 3181–3201 and 3403–3423 from NM_009782. The aliquots of PCR products were electrophoresed on 1·5% agarose gels and visualized after ethidium bromide staining. For the quantification of *Gnrh*, *Erα*, *Erβ*, *Cacna1e*, and *Rpl19* mRNA levels, real-time PCR was performed using LightCycler FastStart DNA master

SYBR Green I system (Roche Diagnostic Co.) under each optimized condition of annealing at 59–63 °C with 4 mM $MgCl_2$, following the manufacturer's protocol. Accumulated levels of fluorescence for each product were analyzed by the second derivative method after the melting curve analysis (Roche Diagnostic Co.), and then, following the assay validation by calculating each amplification efficiency (*Rpl19*, 98·5%; *Gnrh*, 89%; *Erα*, 88%; *Erβ*, 92%; and calcium channel α1E, 105%), the expression levels of target genes were quantified based on standard curve analysis for each product and normalized by *Rpl19* level in each target.

Measurement of GNRH production

To assess the effects of BMPs on GNRH protein synthesis, GT1-7 cells (2×10^5 viable cells/ml) were cultured in 96-well plates with DMEM containing 10% FCS for 24 h. The medium was then changed to serum-free DMEM and subsequently treated with the indicated concentrations of BMPs and E_2. After 24-h culture, the supernatant of the culture media was collected and stored at −80 °C until assay. GNRH concentration (pg/ml) in the conditioned medium was determined by RIA as previously reported (Chappell *et al.* 2003). Briefly, 0·1 ml aliquots of each sample were incubated for 48 h with GNRH antibody EL-14 at 4 °C, after which time 10 000 c.p.m. sample of radio-iodinated GNRH (Amersham Pharmacia) was added. Following ethanol precipitation of the bound fractions after 48-h incubation, radioactivity was detected by a gamma scintillation counter (Micromedic, Huntsville, AL, USA), and the standard curve analysis was performed at each assay. The inter- and intra-assay variability are 8·7 and 7·7% respectively in the current RIA.

Thymidine incorporation assay

GT1-7 cells (1×10^5 viable cells/ml) were precultured in 12-well plates with DMEM containing 10% FCS for 24 h. After preculture medium was replaced with fresh serum-free medium and indicated concentrations of E_2, BMPs and PDGF-BB were added to the culture medium. After 24-h culture, 0·5 μCi/ml [*methyl*-^{3}H] thymidine (Amersham Pharmacia) was added and incubated for 3 h at 37 °C. The incorporated thymidine was detected as previously reported (Takahashi *et al.* 2008). Cells were then washed with PBS, incubated with 10% ice-cold trichloroacetic acid for 60 min at 4 °C, and solubilized in 0·5 M NaOH, and radioactivity was determined with a liquid scintillation counter (TRI-CARB 2300TR, Packard Co., Meriden, CT, USA).

Western immunoblot analysis

GT1-7 cells (1×10^5 viable cells/ml) were precultured in 12-well plates in serum-free DMEM. After 24-h preculture, BMPs and activin A (100 ng/ml) were added to the culture medium either alone or in combination with E_2 (100 nM). After acute (10–60 min) or chronic (24 h) stimulations with indicated concentrations of BMPs in combination with E_2 and various chemical inhibitors including U0126, SB203580, SP600125, and ICI 182 780, cells were solubilized in 100 μl RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY, USA) containing 1 mM Na_3VO_4, 1 mM NaF, 2% SDS, and 4% β-mercaptoethanol.

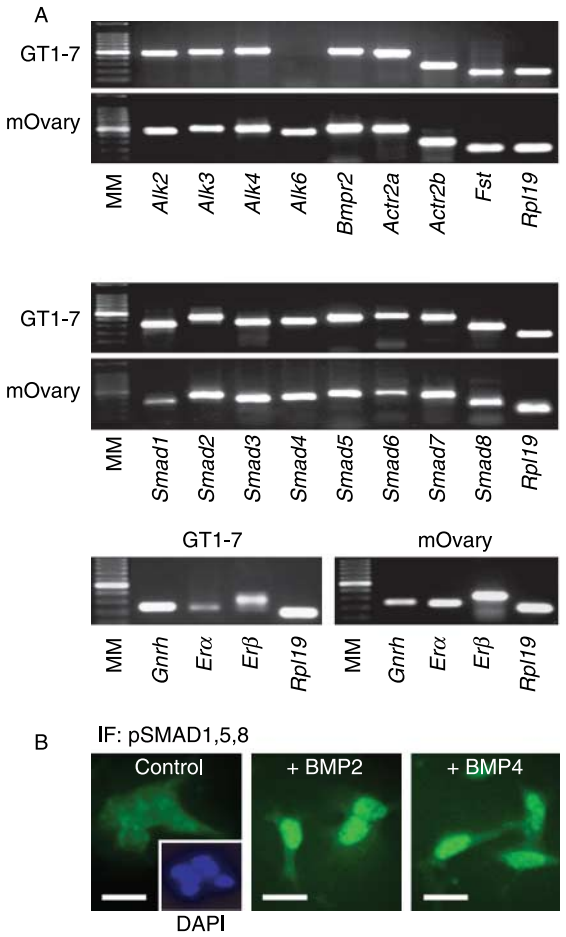

Figure 1 Expression of BMP system and estrogen receptor (ER) in GT1-7 cells. (A) Total cellular RNAs were extracted from GT1-7 cells and quantified by measuring the absorbance of the sample at 260 nm. The expression of mRNAs encoding *Alk-2, -3, -4, -6, Bmpr2, Actr2a, Actr2b, Fst, Smad1–8, Gnrh, Erα, Erβ*, and a housekeeping gene *Rpl19* was examined by RT-PCR analysis in GT1-7 cells compared with a control sample extracted from mouse ovarian tissues. Aliquots of PCR products were electrophoresed on 1·5% agarose gel, visualized by ethidium bromide staining, and shown as representative of those obtained from three independent experiments. MM indicates molecular weight marker. (B) GT1-7 cells were precultured for 24 h and stimulated with BMP-2 and -4 (100 ng/ml) for 1 h. Immunofluorescence (IF) studies were performed using anti-phospho-SMAD1, 5, 8 (pSMAD1, 5, 8) antibody on BMP-treated cells. DAPI indicates counterstaining with 4′,6′-diaminodino-2-phenylindole. Bars indicate 20 μm in size.

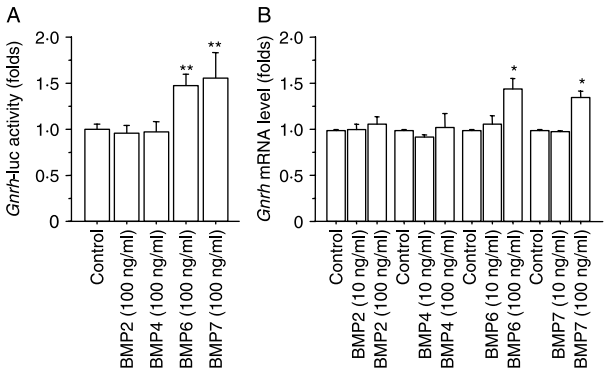

Figure 2 Effects of BMPs on *Gnrh* transcription and *Gnrh* mRNA expression in GT1-7 cells. (A) GT1-7 cells (1×10^5 cells/ml) were transiently transfected with *Gnrh*-luc reporter plasmid (500 ng) and pCMV-β-gal. After 24-h treatment with BMP-2, -4, -6, and -7 (100 ng/ml), cells were lysed and the luciferase activity was measured. The data were analyzed as the ratio of luciferase to β-galactosidase (β-gal) activity. Results are shown as mean±S.E.M. of data from at least three separate experiments, each performed with triplicate samples. $^{**}P<0{\cdot}01$ versus control groups. (B) GT1-7 cells (2×10^5 cells/ml) were treated with BMP-2, -4, -6, and -7 (10 and 100 ng/ml) in serum-free conditions for 24 h. Total cellular RNA was extracted and *Gnrh* mRNA levels were examined by quantitative real-time RT-PCR. The expression levels of target genes were standardized by *Rpl19* level in each sample. Results are shown as mean±S.E.M. of data from at least three separate experiments, each performed with triplicate samples. $^{*}P<0{\cdot}05$ versus control groups.

The cell lysates were then subjected to SDS-PAGE/immunoblotting analysis as previously reported (Inagaki *et al*. 2006, Otani *et al*. 2007), using anti-phospho- and anti-total-extracellular signal-regulated kinase (ERK) 1/2 mitogen-activated protein kinase (MAPK) antibody (Cell Signaling Technology, Inc., Beverly, MA, USA), anti-phospho- and anti-total-p38 MAPK antibody (Cell Signaling Technology, Inc.), anti-phospho- and anti-total-stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK) MAPK antibody (Cell Signaling Technology, Inc.), anti-phospho-Smad1, 5, 8 (pSmad1, 5, 8) antibody (Cell Signaling Technology, Inc.), anti-Smad5 antibody (Cell Signaling Technology, Inc.), anti-actin antibody (Sigma–Aldrich Co. Ltd), and anti-ERα (MC-20), and anti-ERβ (H-150) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Immunofluorescence microscopy

For the immunofluorescence study, GT1-7 cells were precultured in serum-free DMEM using chamber slides (Nalge Nunc Int, Naperville, IL, USA). Cells at ∼50% confluency were treated with BMP-2 and BMP-4 (100 ng/ml) for 1 h. Cells were then fixed with 4% paraformaldehyde in PBS, permeabilized with 0·5% Triton X-100 in PBS at room temperature, and washed thrice with PBS. The cells were then incubated with anti-phospho-SMAD1, 5, 8 antibody (Cell signaling Technology, Inc.) for 1 h and washed thrice with PBS, then with Alexa Fluor 488 anti-rabbit IgG (Invitrogen Corp.) in humidified chamber for 1 h and washed with PBS, followed by application of the counter medium containing 4′,6′-diaminodino-2-phenylindole (Invitorogen Corp.), and then stained cells were visualized under a fluorescent microscope.

Transient transfection and luciferase assay

GT1-7 cells (1×10^5 viable cells/ml) were precultured in 12-well plates in DMEM with 10% FCS for 24 h. The cells were transiently transfected with 500 ng *Gnrh*-luc reporter plasmid, which contains 173-bp rat *Gnrh* promoter region (Nelson *et al*. 2000), and 50 ng cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal) using FuGENE6 (Roche Molecular Biochemicals) for 24 h. The cells were then treated with indicated concentrations of BMPs and E_2 for 24 h in serum-free fresh medium. After 24-h culture, the cells were washed with PBS and lysed with Cell Culture Lysis Reagent (Toyobo, Osaka, Japan). Luciferase activity and β-gal activity of the cell lysate were measured by luminescencer-PSN (ATTO, Tokyo, Japan) as previously reported (Miyoshi *et al*. 2006). The data were shown as the ratio of luciferase to β-gal activity.

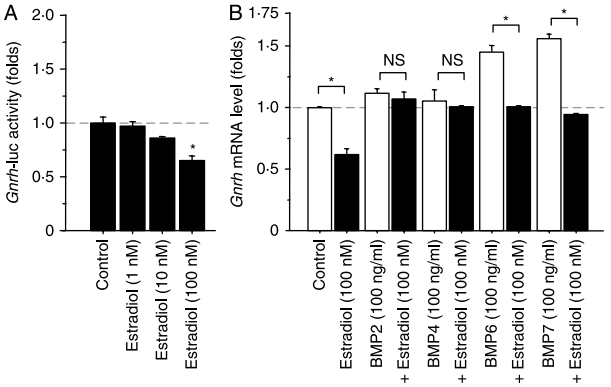

Figure 3 Effects of BMPs and estradiol on *Gnrh* transcription in GT1-7 cells. (A) GT1-7 cells (1×10^5 cells/ml) were transiently transfected with *Gnrh*-luc reporter plasmid (500 ng) and pCMV-β-gal. After 24-h treatment with estradiol (1–100 nM), cells were lysed and the luciferase activity was measured. The data were analyzed as the ratio of luciferase to β-galactosidase (β-gal) activity. Results are shown as mean±S.E.M. of data from at least three separate experiments, each performed with triplicate samples. $^{*}P<0{\cdot}05$ versus control groups. (B) Cells (2×10^5 cells/ml) were treated with BMP-2, -4, -6, and -7 (100 ng/ml) in the presence or absence of estradiol (100 nM) in serum-free conditions for 24 h. Total cellular RNA was extracted and *Gnrh* mRNA levels were examined by real-time RT-PCR. The expression levels of target genes were standardized by *Rpl19* level in each sample. Results are shown as mean±S.E.M. of data from at least three separate experiments, each performed with triplicate samples. $^{*}P<0{\cdot}05$ versus control groups or between the indicated groups; NS, not significant.

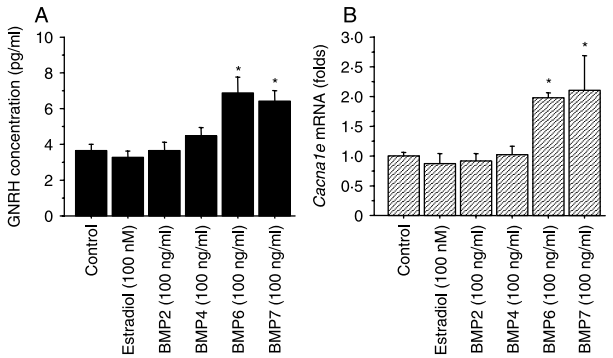

Figure 4 Effects of BMPs on GNRH release by GT1-7 cells. (A) GT1-7 cells (2×10^5 cells/ml) were treated with indicated concentrations of estradiol, BMP-2, -4, -6, and -7 in serum-free conditions. After 24-h culture, the culture media was collected, and GNRH concentrations (pg/ml) were determined by RIA. Results show the mean ± S.E.M. of data performed with triplicate treatments; *$P<0{\cdot}05$ versus control groups. (B) GT1-7 cells were treated with indicated concentrations of estradiol, BMP-2, -4, -6, and -7 in serum-free conditions for 24 h. Total cellular RNA was extracted and mRNA levels of *Cacna1e* were examined by real-time RT-PCR. The expression levels of target genes were standardized by *Rpl19* level in each sample. Results are shown as mean ± S.E.M. of data from at least three separate experiments, each performed with triplicate samples. *$P<0{\cdot}05$ versus control groups.

Statistical analysis

All results are shown as mean ± S.E.M. of data from at least three separate experiments, each performed with triplicate samples. Differences between groups were analyzed for statistical significance using ANOVA with Fisher's protected least significant difference (PLSD) test (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA, USA). *P* values $<0{\cdot}05$ were accepted as statistically significant.

Results

We first examined mRNA expression of the BMP type I and type II receptors in GT1-7 cells by RT-PCR. As shown in Fig. 1A, the BMP type I receptors including activin receptor-like kinase (*Alk2*) (also called *Actr1a*), *Alk3* (*Bmpr1a*), and *Alk4* (*Actr1b*), BMP type II receptors including *Bmpr2*, *Actr2*, and *Actr2b*, and a binding protein *Fst* were clearly expressed in GT1-7 cells. Compared with mouse ovary RNA positive control, the expression of *Alk6* (*Bmpr1b*) was not detected in GT1-7 cells. In addition, mRNA expression of Smad signaling molecules including *Smad1, 2, 3, 4, 5,* and *8*, and inhibitory *Smad6* and 7 was also detected (Fig. 1A). The expression of two subtypes of ERs including *Erα* and *Erβ*, and *Gnrh* mRNA was also confirmed in GT1-7 cells. As shown in Fig. 1B, the immunofluorescence study demonstrated the nuclear localization of phosphorylated SMAD1, 5, 8 molecules in GT1-7 cells stimulated by BMP-2 and -4 (100 ng/ml), indicating that the BMP receptors and SMADs are functionally active in GT1-7 cells.

To investigate the BMP effects on *Gnrh* transcription, *Gnrh*-promoter activity was examined in GT1-7 cells transiently transfected with a *Gnrh*-luc reporter plasmid containing 173-bp rat *Gnrh* promoter region (Nelson *et al.* 2000). In GT1-7 hypothalamic neurons, a neuron-specific enhancer responsible for directing expression of the rat *Gnrh* gene was identified (Whyte *et al.* 1995), in which ~300-bp upstream region confers enhancer activity to a 173-bp *Gnrh* promoter specifically in GT1-7 cells. In the present study, in order to simply examine *Gnrh* promoter activity induced by BMPs, only the key promoter region containing 173-bp rat *Gnrh* promoter was utilized for this promoter assay. As a result, BMP6 and BMP7 stimulated transcriptional activity of *Gnrh* gene, while BMP2 and BMP4 had no significant effects on *Gnrh*-promoter activity (Fig. 2A). To further examine the BMP effects on steady-state *Gnrh* mRNA levels in GT1-7 cells, cells were treated with BMP ligands for 24 h in serum-free conditions. Treatment with BMP2 and BMP4 (10 and 100 ng/ml) had no effect on *Gnrh* mRNA expression by GT1-7 cells (Fig. 2B). In accordance with the results of *Gnrh* promoter assay, BMP6 and BMP7 (10 and 100 ng/ml) significantly increased *Gnrh* mRNA expression (Fig. 2B).

In addition, treatment with E_2 (1–100 nM) significantly decreased *Gnrh*-luc activity (Fig. 3A) and *Gnrh* mRNA expression levels (Fig. 3B). Notably, E_2 (100 nM)-induced reduction of *Gnrh* mRNA was reversed by treatment with BMP2 and BMP4. In contrast, E_2-induced *Gnrh* suppression was not apparently affected by treatment with BMP6 and BMP7 (100 ng/ml; Fig. 3B).

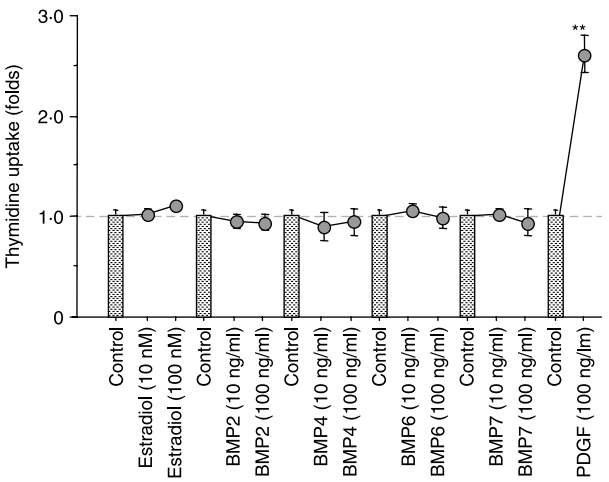

Figure 5 Effects of BMPs and estradiol on GT1-7 cell proliferation. GT1-7 cells (1×10^5 cells/ml) were treated with indicated concentrations of estradiol, BMP-2, -4, -6, -7, and PDGF-BB for 24 h in serum-free condition, and then thymidine uptake assay was performed. Results are shown as mean ± S.E.M. of data from at least three separate experiments, each performed with triplicate samples. **$P<0{\cdot}01$ versus control groups.

The effects of BMPs on GNRH production were evaluated by measuring GNRH concentration in the conditioned medium by RIA (Fig. 4A). In accordance with the results of *Gnrh* transcription, BMP6 and BMP7 increased GNRH concentration (pg/ml) by GT1-7 cells, while E_2, BMP2, and BMP4 had no effects on the GNRH levels (Fig. 4A). To investigate the involvement of BMPs in GNRH release by GT1-7 cells, the expression changes in a key calcium channel, α1E-subunit were examined (Watanabe *et al.* 2004). Various stimuli including potassium and acetylcholine have known to induce GNRH secretion through increased influx of intracellular calcium by GT1-7 cells. The α1E-subunit of R-type calcium channel was recently shown to be critical for GNRH secretion in GT1-7 cells (Watanabe *et al.* 2004). BMP6 and BMP7 increased mRNA expression of α1E-subunit of R-type calcium channel, although E_2, BMP2, and BMP4 had no effects on α1E-subunit mRNA (Fig. 4B). In addition, the levels of thymidine incorporation in GT1-7 cells were not increased in response to treatment with E_2, BMP-2, -4, -6, and -7, although GT1-7 cells were responsive to exogenous PDGF-BB (Fig. 5). It is thus possible that BMP6 and BMP7 may directly enhance GNRH release through up-regulating a critical calcium channel expression in GT1-7 cells.

Western blots also showed SMAD1, 5, 8 signaling activation by BMP-2, -4, -6, and -7 in GT1-7 cells (Fig. 6A). Treatments with BMP-2, -4, -6, and -7, in particular BMP2 and 4, activated Smad1, 5, 8 phosphorylation in GT1-7 cells (Fig. 6B). The BMP-induced SMAD1, 5, 8 activation was not affected in the presence of E_2 (100 nM). It was of note that treatment with E_2 (100 nM) significantly activated MAPK phosphorylation including ERK1/ERK2 and SAPK/JNK but not p38-MAPK signaling (Fig. 6A). BMP-2 and BMP-4 significantly suppressed E_2 (100 nM)-induced phosphorylation of ERK1/ERK2 and SAPK/JNK signaling, whereas BMP6 and BMP7 did not affect E_2-induced activation of ERK1/ERK2 and SAPK/JNK (Fig. 6B). Activin A (100 ng/ml) induced p38 phosphorylation in the presence or absence of E_2, while activin A did not affect E_2-induced ERK1/ERK2 activation.

Since treatment with E_2 activated MAPK pathways including ERK1/ERK2 and SAPK/JNK signaling, the functional roles of these pathways in *Gnrh* transcription were examined. Treatment with 3 μM U0126 specifically inhibited basal and E_2-induced ERK1/ERK2 phosphorylation (Fig. 7A). SP600125 (3 and 10 μM) inhibited basal and E_2-induced SAPK/JNK phosphorylation in GT1-7 cells,

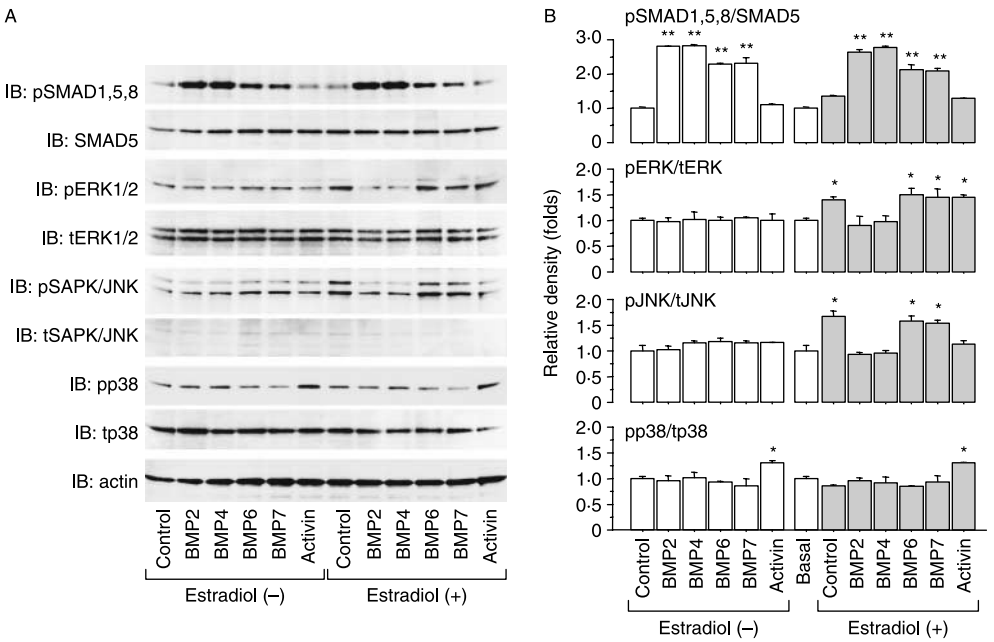

Figure 6 Effects of BMPs and estradiol on SMAD/MAPK signaling in GT1-7 cells. (A) Cells (1×10^5 cells/ml) were precultured for 24 h in serum-free condition and stimulated with BMP-2, -4, -6, -7, and activin A (100 ng/ml) in the absence or presence of estradiol (100 nM). After 60-min culture, cells were lysed and subjected to SDS-PAGE/immunoblotting (IB) analysis using anti-phospho-SMAD1, 5, 8 (pSMAD1, 5, 8) and anti-SMAD5, anti-phospho-ERK1/2 (pERK1/2) and anti-total-ERK1/2 (tERK1/2), anti-phospho-SAPK/JNK (pSAPK/JNK) and anti-total-SAPK/JNK (tSAPK/JNK), anti-phospho-p38 (pp38) and anti-total-p38 (tp38), and anti-actin antibodies. The results shown are representative of those obtained from three independent experiments. (B) The relative integrated density of each protein band of Fig. 6A was digitized by NIH image J 1.34s. Results are shown as mean ± S.E.M. of data from at least three separate experiments, each performed with triplicate samples. $^{**}P<0{\cdot}01$ and $^{*}P<0{\cdot}05$ versus control or basal levels.

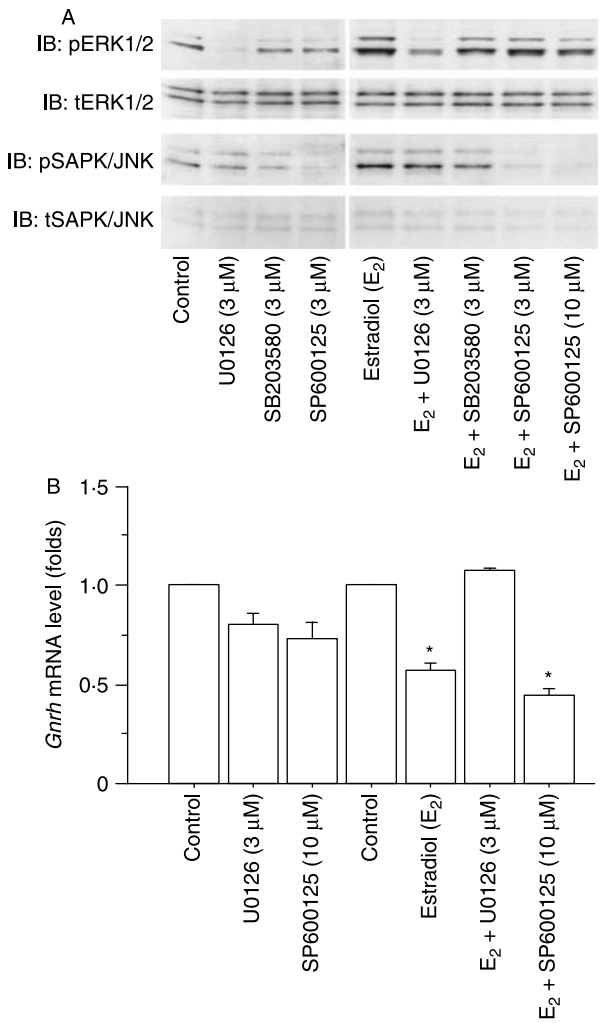

Figure 7 Effects of MAPK inhibition on *Gnrh* regulation by GT1-7 cells. (A) GT1-7 cells (1×10^5 cells/ml) were precultured for 24 h and then treated with MAPK inhibitors including U0126, SB203580, and SP600125 (3–10 μM) in combination with estradiol (E_2; 100 nM). After 60-min culture, cells were lysed and subjected to SDS-PAGE/immunoblotting (IB) analysis using anti-phospho-ERK1/2 (pERK1/2), anti-total-ERK1/2 (tERK1/2), anti-phospho-SAPK/JNK (pSAPK/JNK), and anti-total SAPK/JNK (tSAPK/JNK) antibodies. The results shown are representative of those obtained from three independent experiments. (B) GT1-7 cells (2×10^5 cells/ml) were treated with U0126 (3 μM) and SP600125 (10 μM) in the absence or presence of estradiol (100 nM) in serum-free conditions for 24 h. Total cellular RNA was extracted and *Gnrh* mRNA levels were examined by real-time RT-PCR. The expression levels of target genes were standardized by *Rpl19* level in each sample. Results are shown as mean ± S.E.M. of data from at least three separate experiments, each performed with triplicate samples. *$P<0{\cdot}05$ versus control groups.

although SB203580 (3 μM) did not affect either ERK1/ERK2 or SAPK/JNK phosphorylation (Fig. 7A). As shown in Fig. 7B, U0126 (3 μM) restored the E_2-induced suppression of *Gnrh* expression, although either U0126 or SP600125 alone had no significant effects on *Gnrh* expression in GT1-7 cells. In contrast, SP600125 (10 μM) failed to reverse the inhibitory effect of estrogen on *Gnrh* expression. Thus, the effects of estrogen on *Gnrh* suppression occur through ERK1/ERK2 activation in GT1-7 cells.

We next investigated whether the E_2 actions on MAPK activation and *Gnrh* suppression are mediated via ER. As shown in Fig. 8A, ERK1/ERK2 phosphorylation induced by E_2 (100 nM) was inhibited in the presence of a competitive ER antagonist ICI 182 780 (30–300 nM), although ICI 182 780 alone had no effects on ERK phosphorylation. A membrane-impermeable E_2-BSA (100 nM) formulation failed to activate ERK1/ERK2 pathway (Fig. 8A). Notably, treatment with ICI 182 780 reversed the E_2-induced suppression of *Gnrh* mRNA, whereas ICI 182 780 (30 nM) or E_2-BSA (100 nM) alone did not change the *Gnrh* expression in GT1-7 cells (Fig. 8B). These suggest that estrogen inhibits *Gnrh* expression in GT1-7 cells at least in part by activating ERK1/ERK2 pathway via classic ER. Given the finding that BMP2 and BMP4 suppressed estrogen-induced phosphorylation of MAPK signaling, BMP2 and BMP4 may regulate the ER activity leading to GNRH reduction in GT1-7 cells.

To further investigate the involvement of ER in BMP-induced *Gnrh* regulation, the expression levels of *Erα* and *Erβ* mRNA were examined in GT1-7 cells treated with BMPs and E_2. BMP2 and BMP4 (10–100 ng/ml) significantly decreased mRNA levels of *Erα* and *Erβ*, while BMP6 and BMP7 increased the expression of *Erα* and *Erβ* (Fig. 9A and B). E_2 (100 nM) itself also decreased *Erα* mRNA levels in GT1-7 cells. In addition, the changes of ERα and ERβ protein levels were examined by western immunoblotting in GT1-7 cell lysates treated with BMP-2, -4, -6, and -7 for 24 h. As shown in Fig. 9C, the expression levels of ERα (66 kDa) and ERβ (56 kDa) proteins were decreased by BMP2 and BMP4, while BMP6 and BMP7 in turn increased ERα and ERβ protein expression, although the levels of an internal control actin (42 kDa) were unchanged in GT1-7 cells. Thus, BMP2 and BMP4 are likely to downregulate estrogen effects by attenuating estrogen-induced ERK1/ERK2 activation and by suppressing the expression of ER in GT1-7 cells.

Discussion

The episodic secretion of pituitary gonadotropin is essential for the maintenance of the mammalian reproductive cycle and normal gonadal function. In the present study, we showed that hypothalamic GT1-7 neuron cells express functional machinery of the BMP system, and that the BMP system acts to modulate estrogen effects on *Gnrh* regulation. E_2 suppressed GNRH production by activating the MAPK pathway through ER action. In this feedback loop between E_2 and GNRH, BMP2 and BMP4 suppress estrogen effects by attenuating estrogen ERK signaling with reduction of ERα and ERβ expression. In contrast, BMP6 and BMP7 directly

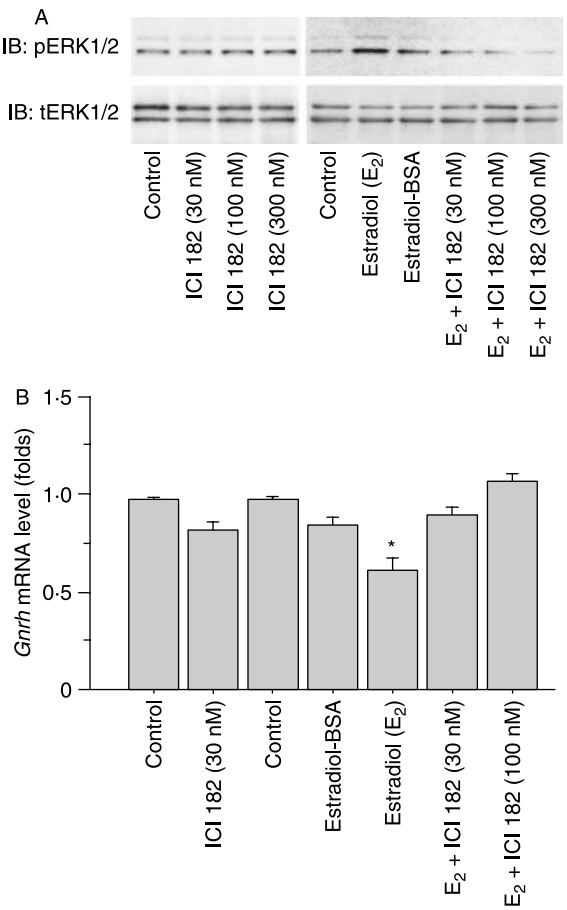

Figure 8 Effects of estrogen receptor (ER) inhibition on *Gnrh* regulation by GT1-7 cells. (A) GT1-7 cells (1×10^5 cells/ml) were precultured for 24 h and then treated with ICI 182 780 (30–300 nM), estradiol (E_2; 100 nM), and estradiol-BSA (100 nM). After 60-min culture, cells were lysed and subjected to SDS-PAGE/immunoblotting (IB) analysis using anti-phospho-ERK1/2 (pERK1/2) and anti-total-ERK1/2 (tERK1/2) antibodies. The results shown are representative of those obtained from three independent experiments. (B) GT1-7 cells (2×10^5 cells/ml) were treated with ICI 182 780 (30–100 nM), estradiol (E_2; 100 nM), and estradiol-BSA (100 nM) in serum-free conditions for 24 h. Total cellular RNA was extracted and *Gnrh* mRNA levels were examined by real-time RT-PCR. The expression levels of target genes were standardized by *Rpl19* level in each sample. Results are shown as mean±S.E.M. of data from at least three separate experiments, each performed with triplicate samples. $^*P<0{\cdot}05$ versus control groups.

stimulated *Gnrh* transcription and secretion by GT1-7 cells. This novel interaction of BMPs and ER action is likely involved in controlling hypothalamic GNRH release in an autocrine/paracrine mechanism (Fig. 10).

Estrogen has rapid regulatory effects on membrane-associated and intracellular responses (often referred to as nongenomic actions), in addition to the well-defined genomic actions in the nucleus. In the present study, E_2 readily activated MAPK phosphorylation including ERK1/ERK2 and SAPK/JNK but not p38-MAPK signaling, implying the involvement of nongenomic effects of estrogen in GT1-7 cells. BMP2 and BMP4 suppressed the estrogen-induced phosphorylation of ERK1/ERK2 and SAPK/JNK signaling, whereas BMP6 and BMP7 did not affect the E_2-induced activation of ERK1/ERK2 and SAPK/JNK. Since the inhibition of ERK1/ERK2 restored the E_2-induced suppression of *Gnrh* expression by GT1-7 cells, the effects of estrogen on *Gnrh* suppression is likely to occur through ERK1/ERK2 pathway in GT1-7 cells.

These estrogen effects may be distinct from the genomic actions of estrogen and are attributable to its interactions with membrane-bound receptors and other cellular components (Pietras & Szego 1999, Pietras *et al.* 2005). Membrane ER has been observed in the hypothalamus and other brain regions, including the cortex, hippocampus, and brain stem. The extent to which GNRH neurons are directly responsive to steroid hormones has been controversial. However, it is now being accepted that hypothalamic cells express both membrane-associated and nuclear receptors, and that they are capable of directly responding to changes in systemic estrogen (Malyala *et al.* 2005).

In our data, ERK1/ERK2 phosphorylation induced by E_2 was inhibited in the presence of ICI 182 780, an ER antagonist that inhibits both ERα and ERβ activities. In addition, the effects of membrane-impermeable E_2-BSA were much less effective than E_2 for activating ERK1/ERK2 signaling in GT1-7 cells. ICI 182 780 reversed the E_2-induced suppression of *Gnrh* mRNA and E_2–BSA failed to suppress *Gnrh* expression in GT1-7 cells. These findings suggest that estrogen inhibits *Gnrh* expression, at least in part, by activating ERK1/ERK2 pathway via classic ER signaling. Given the finding that BMP2 and BMP4 suppressed estrogen-induced phosphorylation of MAPK signaling, including ERK1/ERK2, BMP2, and BMP4, may directly regulate the ER activity leading to GNRH reduction in GT1-7 cells.

In agreement with our present results, Roy *et al.* (1999) demonstrated that E_2 represses *Gnrh* gene expression in GT1-7 cells. This estrogen effect was blocked by ICI 182 780 and also mimicked by 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane, a putative ERα agonist and ERβ antagonist (Gaido *et al.* 1999, Roy *et al.* 1999), suggesting that ERα may be responsible for the repressive effects of E_2 on *Gnrh* expression. However, a phytoestrogen, coumestrol, also suppressed *Gnrh* expression, which was in turn blocked by an ERβ antagonist, *R,R*-tetrahydrochrysene (Bowe *et al.* 2003). These results also imply that estrogen negatively regulates *Gnrh* promoter activity via not only ERα but also ERβ in hypothalamic cells. In the present study, the expression levels of both *Erα* and *Erβ* were decreased in GT1-7 cells treated with BMP2 and BMP4, which could be linked to the suppression of estrogen-induced MAPK activation. To the contrary, the expression levels of *Erα* and *Erβ* were increased by BMP6 and BMP7. This effect may be associated with the upregulation of estrogen–GNRH feedback function following the increase in GNRH production caused by BMP6 and BMP7.

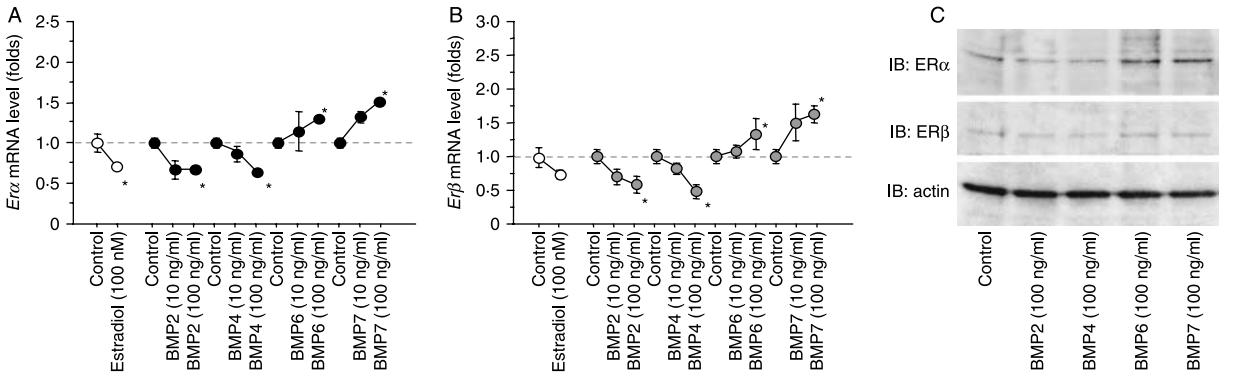

Figure 9 Effects of BMPs and estradiol on estrogen receptor (*Er*) expression in GT1-7 cells. (A and B) GT1-7 cells were treated with BMP-2, -4, -6, and -7 (10 and 100 ng/ml) and estradiol (100 nM) in serum-free conditions for 24 h. Total cellular RNA was extracted and *Erα* and *Erβ* mRNA levels were examined by real-time RT-PCR. The expression levels of target genes were standardized by *Rpl19* level in each sample. Results are shown as mean ± S.E.M. of data from at least three separate experiments, each performed with triplicate samples. $^{*}P<0{\cdot}05$ versus control groups. (C) Cells (1×10^5 cells/ml) were treated with BMP-2, -4, -6, and -7 (100 ng/ml) in serum-free condition. After 24-h culture, cells were lysed and subjected to SDS-PAGE/immunoblotting (IB) analysis using anti-ERα, anti-ERβ, and anti-actin antibodies. The results shown are representative of those obtained from three independent experiments.

The mechanism whereby estrogen regulates GNRH secretion may be partly due to an indirect effect on the neighboring neurons, wherein estrogen acts in part to stimulate the GNRH and LH surge by turning off inhibitory neurons and turning on excitatory neurons. The principal determinants of GNRH neuronal activity include gonadal steroids, various neuropeptides, and neurotransmitters. In this regard, work from several laboratories has shown that hypothalamic astrocytes release the factors that can stimulate GNRH release (Gallo & Russell 1995, Melcangi *et al.* 1995*a*, Avola *et al.* 2000, Buchanan *et al.* 2000, Ojeda *et al.* 2000, Dhandapani *et al.* 2003). Conditioned media collected from purified hypothalamic astrocytes markedly stimulated GNRH release from GT1-7 neurons, an immortalized GNRH neuronal cell line (Buchanan *et al.* 2000). A similar GNRH stimulatory effect has been reported with conditioned media from cerebral cortical astrocytes (Melcangi *et al.* 1995*b*). In our current study, E_2 suppressed *Gnrh* transcription, whereas GNRH protein concentration was not significantly changed in the culture medium. The suppression of *Gnrh* mRNA was observed in GT1-7 cells in our data and by others (Roy *et al.* 1999). Regarding GNRH secretion, Navarro *et al.* (2003) reported that GNRH release was not affected by picomolar of estrogen in static culture of GT1-7 cells and hypothalamic cells, which was rather up-regulated in the presence of cyclic treatment with progesterone. Given that E_2 did not affect the expression of a key calcium channel for GNRH secretion in GT1-7 cells, E_2 may not directly regulate the GNRH release at least for a certain culture period regardless of the reduction of *Gnrh* transcription. The discrepancy between transcriptional regulation and secretory process of GNRH needs to be elucidated in a future study.

It has been reported that TGF-β1 as a neuroregulatory factor secreted by astrocytes can modulate GNRH secretion (Melcangi *et al.* 1995*b*, Galbiati *et al.* 1996, Buchanan *et al.* 2000, Zwain *et al.* 2002). Additionally, Ojeda *et al.* have provided evidence in support of a neuroregulatory role for astrocyte-derived TGF-α (Ma *et al.* 1992, 1997, Ojeda *et al.* 2000). Furthermore, neuroactive steroid metabolites such as 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP) are also known to be produced by astrocytes, which are capable of stimulating the release of GNRH both *in vitro* and *in vivo* (el-Etr *et al.* 1995, Sim *et al.* 2001). Given the findings that several BMPs share a neurotropic capacity for dopaminergic neurons with other members of the TGF-β superfamily through glial cells (Jordan *et al.* 1997, Reiriz *et al.* 1999, Samanta *et al.* 2007), it is presumable that hypothalamic

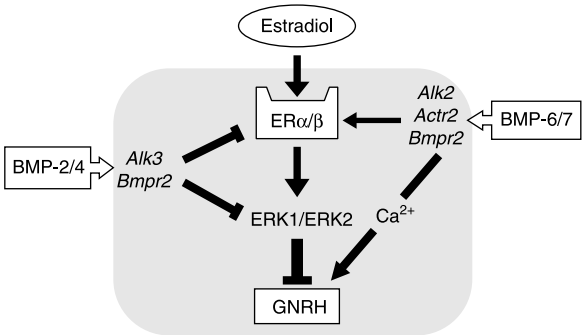

Figure 10 A possible mechanism by which BMPs and estrogen regulate GNRH production by GT1-7 cells. Estradiol suppresses GNRH production by inhibiting ERK1/ERK2 pathway via ER. In this feedback system between estradiol and GNRH production, BMP2 and BMP4 downregulate estrogen effects by attenuating estrogen-ERK signaling and ERα and ERβ expression. In contrast, BMP6 and BMP7 directly stimulate *Gnrh* transcription, GNRH production, and GNRH release via increased expression of α1E-subunit of R-type calcium channel, which is critical for GNRH secretion. A new interaction of BMPs and ER action may be involved in controlling hypothalamic GNRH production and secretion in an autocrine/paracrine mechanism.

GNRH production and/or secretion may also be indirectly regulated by BMP system in the neural network.

In addition, GT1-7 cells generate spontaneous action potentials, exhibit transient oscillations of the intracellular calcium concentration, and secrete GnRH in a pulsatile manner (Charles & Hales 1995, Sun *et al*. 1998, Morales *et al*. 2005). The expression of the R-, L-, N-, and T-type calcium channels was demonstrated in GT1-7 cells, in which the R-type subunit plays a key role as a major current component for regulating calcium-dependent GNRH release compared with L-, N-, and T-type channels (Watanabe *et al*. 2004). Since BMP6 and BMP7 increased mRNA expression of α1E-subunit of R-type calcium channel, which is a critical subunit for hypothalamic GNRH secretion, it seems likely that BMP6 and BMP7 directly stimulate GNRH release by GT1-7 cells. Further investigation would be necessary to clarify whether BMP6 and BMP7 physiologically stimulate calcium channel activity in GNRH neural cells in the future study.

Recent studies have shown that BMPs regulate the pituitary–gonadal endocrine axis. However, roles of BMPs in hypothalamic GNRH production have yet to be clarified. Understanding the neural mechanisms through which E_2 regulates GNRH neurons is key for elucidating reproductive control of the HPO axis. We uncovered here the effects of BMPs on GNRH regulation controlled by estrogen in hypothalamic neuron cells. In particular, BMP2 and BMP4 down-regulate estrogen effects on suppressing GNRH production by attenuating estrogen ERK signaling and by suppressing ER expression. On the contrary, BMP6 and BMP7 contribute to increase in GNRH secretion. Hence, a new interaction of BMPs and ER may be involved in controlling hypothalamic GNRH production and secretion in an autocrine/paracrine mechanism (Fig. 10). The BMP system may play a key role in regulating not only in the ovarian folliculogenesis by regulating gonadotropin sensitivity but also in modulating hypothalamic GNRH secretions leading to fine-tuning of gonadotropin secretion from the pituitary gonadotrope.

Declaration of interest

All authors have nothing to disclose.

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