Orexin A modulates prolactin production by regulating BMP-4 activity in rat pituitary lactotrope cells.

Satoshi Fujisawa¹, Motoshi Komatsubara¹, Kanako Ogura-Ochi¹, Naoko Tsukamoto-Yamauchi¹, Kishio Toma¹, Kenichi Inagaki¹, Jun Wada¹ and Fumio Otsuka²

¹Department of Nephrology, Rheumatology, Endocrinology and Metabolism and ²Department of General Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

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Abbreviations: AC, adenylyl cyclase; ActRII, activin type II receptor; ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; BMPRII, BMP type II receptor; Dor, dorsomorphin; DORA, dual ORX receptor antagonist; ER, estrogen receptor; FSH, follicle-stimulating hormone; FSK, forskolin; LDN, LDN193189; ORX, orexin A; OX1R, orexin type 1 receptor; OX2R, orexin type 2 receptor; and PRL, prolactin.

Correspondence to: Fumio OTSUKA, M.D., Ph.D.
Department of General Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kitaku, Okayama 700-8558, Japan. Phone: +81-86-235-7342, Fax: +81-86-222-7345 E-mail: fumiotsu@md.okayama-u.ac.jp
Abstract

The impact of orexins on anterior pituitary function has yet to be clarified. We studied the effects of orexin A and its interaction with the bone morphogenetic protein (BMP) system on the regulatory role of prolactin synthesis using rat lactotrope GH3 cells expressing BMP-4. Orexin type 1 receptor (OX1R), but not type 2 receptor (OX2R), was predominantly expressed in GH3 cells. Orexin A suppressed forskolin-induced, but not basal, prolactin mRNA expression without reducing cAMP levels. Of note, orexin A suppressed BMP-4-induced prolactin mRNA and cAMP synthesis. Impairment of the effects of orexin by chemical inhibitors suggested involvement of the P38 pathway in the OX1R activity that suppresses BMP-4-induced PRL expression. Given that inhibition of BMP-receptor signaling reduced prolactin mRNA levels, endogenous BMP action is likely to be linked to the activation of prolactin synthesis by GH3 cells. Orexin A was revealed to suppress Smad1/5/9 phosphorylation and Id-1 transcription induced by BMP-4, which was restored in the presence of orexin-receptor antagonists, suggesting that the inhibitory effect of orexin A occurred via OX1R. Orexin A also reduced ALK-3 expression but increased inhibitory Smad6/7 expression, while BMP-4 treatment downregulated OX1R expression. These results indicated that orexin A plays an inhibitory role in prolactin production through suppression of endogenous BMP activity in GH3 cells, suggesting that a new functional role of the interaction between orexin and BMP-4 is modulation of prolactin levels in lactotrope cells.
Introduction

Orexins, including orexin A and orexin B, are neuropeptides that are mainly synthesized in the hypothalamus and produced from the common precursor preproorexin, [1, 2]. Orexins have specific affinities to two different receptors coupled with G-proteins, named orexin type 1 and orexin type 2 receptors (OX1R and OX2R). OX1R selectively binds orexin A, while OX2R can bind both orexin A and orexin B with similar affinities [3]. Orexins have important functions in the regulation of sleep-wake balance, energy expenditure and intake of food. The expression of orexins and their receptors has been found in various peripheral tissues [4] and also in many endocrine tissues such as the adrenal, testis, ovary and pituitary [5].

Orexins are functionally linked to the regulation of endocrine activities including the hypothalamic-pituitary-adrenal and -gonadal axes [6, 7] and growth hormone control [8]. Orexin receptors were shown to be expressed in the rat [9], porcine [10] and human pituitaries [11]; however, the effects of orexin on prolactin (PRL) synthesis and the regulatory roles in lactotorope cells have not been clarified. Intra-ventricular administration of orexin reduced the plasma concentration of PRL in experiments using rats [12, 13], and the action of orexin was shown to be mediated by tubero-infundibular dopaminergic neurons (TIDA) in the hypothalamus [14].

There has been accumulating evidence that bone morphogenetic proteins (BMPs), produced as autocrine and/or paracrine factors, play key roles in the differentiation of pituitary cells [15-18]. Interestingly, BMP-4, which induces key
organogenetic actions in the anterior pituitary, has also been shown to have various functions in the pathogenesis of differentiated pituitary tumors including corticotropinomas [16] and prolactinomas [19-21]. It has also been revealed that there is a functional interrelationship between the BMP-4-Smad pathway and estrogen receptor (ER) signaling in the pathogenesis of PRL-producing pituitary adenomas and in the regulation of PRL transcription [22, 23].

In the present study, we studied the activities of orexin by focusing on BMP-4, which can facilitate PRL production and is also involved in the aggressiveness of prolactinomas, in regard to the modulation of PRL secretion by lactotrope cells. A modulatory effect of orexin on PRL production and mutual interaction of orexin and the BMP system were revealed.

Materials and Methods

Experimental Reagents

A mixture of Dulbecco’s Modified Eagle’s Medium/Ham F-12 medium (DMEM/F12), forskolin (FSK), 3-isobutyl-1-methylxanthine (IBMX) and H-89 (cAMP-dependent protein kinase inhibitor) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Recombinant human BMP-4 from R&D Systems Inc. (Minneapolis, MN) and the BMP-receptor signaling inhibitors LDN193189, from Stemgent (San Diego, CA), and dorsomorphin, from Calbiochem (San Diego, CA), were used in the experiments. The ERK inhibitor U0126 and the P38-MAPK inhibitor SB203580 were from Promega Corp. (Madison, WI), and the stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) inhibitor
SP600125 was from Biomol Lab. Inc. (Plymouth Meeting, PA). Human orexin A was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), the dual orexin-receptor antagonist DORA-12 [24] was provided by Merck Sharp & Dohme Corp. (Rahway, NJ), and the selective non-peptide OX1R antagonist SB408124 [25] was purchased from Tocris Bioscience (Bristol, UK).

**GH3 cell culture and measurement of cAMP levels**

Rat pituitary tumor GH3 cells (5 × 10^4 viable cells), originally provided by Prof. Joseph A. Majzoub, Children's Hospital, Harvard Medical School, were cultured in DMEM/F12 supplemented with 10% fetal calf serum (FCS) and antibiotics in 24-well plates under a 5% CO_2 atmosphere at 37°C. After preculture with the growth medium, the medium was changed to a serum-free medium containing 0.1 mM IBMX, an inhibitor of phosphodiesterase activity, and the GH3 cells were treated with the indicated concentrations of orexin A in combination with FSK or BMP-4 for 24 h. The culture medium was then collected and the supernatant of the centrifuged medium was collected and stored at -80°C until measurement. The extracellular contents of cAMP were determined by an enzyme immunoassay (ELISA; Enzo Life Sciences, Inc., NY, USA).

**RNA extraction, reverse transcription (RT) and real-time PCR**

After preculture, cells (1 × 10^5 viable cells) were treated with the indicated concentrations of orexin A in combination with FSK or BMP-4 in the presence or absence of LDN193189, dorsomorphin, orexin receptor inhibitors (DORA-12 and
SB408124), MAPK inhibitors (U0126, SB203580 and SP600125) or H-89 in 12-well plates in a serum-free condition for 24 h. The culture medium was then removed and total cellular RNA was extracted using TRI Reagent® (Cosmo Bio Co., Ltd., Tokyo, Japan). Whole tissues of the normal rat pituitary were obtained from 8-week-old female Wistar rats and total RNA was also extracted using TRI Reagent®. Primer pairs for PCR were selected from different exons of the corresponding genes as follows: rat PRL: 271-291 and 471-491 (NM_012629); rat Id-1: 218-240 and 357-377 (NM_012797); rat OX1R: 1658-1680 and 2057-2079 (NM_013064); rat OX2R: 209-233 and 539-558 (NM_013074); rat BMP-4: 500-521 and 705-724 (NM_012827.2); and rat BMP type II receptor (BMPRII): 1785-1804 and 1942-1961 (NM_080407). Rat Smad6, Smad7, ALK-2, ALK-3 and ribosomal protein L19 (RPL19) were selected as we reported previously [26]. The extracted RNA (1 μg) was subjected to an RT reaction using ReverTra Ace® (TOYOBO CO., LTD., Osaka, Japan) with a random hexamer and deoxynucleotide triphosphate (dNTP). After optimizing the annealing conditions for each pair of primers, quantitative PCR (qPCR) was performed to quantify the level of target gene mRNA using the LightCycler® Nano real-time PCR system (Roche Diagnostic Co., Tokyo, Japan). The relative expression of each mRNA was determined by the ΔCt method, in which ΔCt was the value obtained by subtracting the Ct value of RPL19 mRNA from that of the target mRNA. The amount of target mRNA relative to RPL19 mRNA was expressed as $2^{-\Delta\text{Ct}}$, and the results were expressed as the ratio of target mRNA to RPL19 mRNA.

*Western blots*
GH3 cells (1 × 10^5 viable cells) were precultured with DMEM/F12 containing 10% FCS and antibiotics in 12-well plates. The growth medium was then changed to a serum-free medium and treated with the indicated concentrations of orexin A for 24 h. Subsequently, the cells were stimulated with indicated concentrations of BMP-4 for 60 to 120 min. The treated cells were solubilized in 100 μl RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY) containing 1 mM Na_3VO_4, 1 mM NaF, 2% SDS, and 4% β-mercaptoethanol. Western blot analysis was performed using the cell lysates with specific antibodies against phospho-Smad1/5/9 (pSmad1/5/9), total-Smad1 (tSmad1; Cell Signaling Technology, Inc., Beverly, MA) and actin (Sigma-Aldrich Co. Ltd.). The blotted bands were analyzed by the C-DiGit® Blot Scanner System (LI-COR Biosciences, NE) by scanning the integrated signal intensities. For evaluating the phosphorylated Smad contents, the ratios of the digitized levels of pSmad/tSmad bands were calculated.

Statistics

Experimental results are shown as means ± SEM of data from at least three independent experiments, each performed with triplicate samples. The data were then subjected to ANOVA followed by Tukey-Kramer’s post hoc test or unpaired t-test (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). P values <0.05 were accepted as statistically significant.

Results
Firstly, we assessed the expression of orexin receptors in rat whole pituitary tissues and GH3 cells. The expression of both OX1R and OX2R was detected in the rat whole pituitary by RT-PCR as shown in Fig. 1A, whereas the major orexin receptor for GH3 cells was found to be OX1R. In GH3 cells, BMP-4 expression was also detected by RT-PCR as seen in the rat whole pituitary (Fig. 1A). Treatment with orexin A (10-300 nM) did not change basal mRNA levels of PRL for 24 h as shown in Fig. 1B. FSK (0.3 μM) treatment significantly enhanced mRNA expression of PRL, which was significantly suppressed by co-treatment with orexin A (100 to 300 nM). To investigate the mechanism by which orexin A suppressed FSK-induced PRL synthesis, changes of cAMP levels were examined. As shown in Fig. 1C, orexin A failed to suppress basal and FSK (0.01-0.3 μM)-induced cAMP synthesis.

PRL synthesis is known to be induced by BMP-4 treatment in lactotrope cells, and it is thought that crosstalk between Smad and ER complex [23] and increase in cellular cAMP induction [19] are involved. We therefore examined the functional interrelationship between BMP-4 and orexin in GH3 cells. As shown in Fig. 2A, treatment with BMP-4 (10 ng/ml) significantly enhanced PRL mRNA expression and co-treatment with orexin A (100 nM) suppressed the enhancement of PRL expression. Furthermore, treatment with orexin A significantly reduced BMP-4-induced cAMP levels in 24-h culture, while orexin A failed to suppress basal cAMP synthesis (Fig. 2B). To examine the role of the activity of endogenous BMP expressed in GH3 cells [26], a BMP-receptor signal inhibitor, dorsomorphin, that selectively inhibits ALK-2, -3 and -6 signaling [27] and another inhibitor, LDN193189, that specifically inhibits ALK-2 and -3 actions
were used to inhibit endogenous BMP actions. As shown in Fig. 2C, co-treatment with LDN193189 or dorsomorphin (100-300 nM) reduced basal and FSK-induced levels of PRL mRNA in 24-h culture, indicating that endogenous BMPs augment PRL synthesis by GH3 cells.

Furthermore, to determine the possible signaling pathways of orexin for suppressing BMP-4-induced PRL expression, specific inhibitors (1 µM) for cAMP-PKA and MAPKs were used for examining the changes of PRL mRNA levels. As shown in Fig. 2D, treatment with a P38 inhibitor, SB203580, abolished the suppressive effect of orexin A on BMP-4-induced PRL expression in 24-h culture, whereas treatments with a cAMP-PKA inhibitor, H-89, an ERK inhibitor, U0126, and a SAPK/JNK inhibitor, SP600125, did not affect the action of orexin. These results suggested that P38 signaling is, at least in part, involved in the orexin receptor activity for regulating BMP-4-induced PRL expression in GH3 cells.

Next, we examined the involvement of orexin action in the intracellular signaling of BMP-4. Stimulation with BMP-4 (10 ng/ml) for 2 h readily activated Smad1/5/9 phosphorylation in GH3 cells as shown in Fig. 3A. Importantly, orexin A (100 nM) pretreatment for 24 h suppressed Smad1/5/9 phosphorylation induced by BMP-4 (Fig. 3A), suggesting that orexin A suppresses PRL production by reducing Smad1/5/9 activation in GH3 cells. To clarify the effects of the orexin receptor on BMP-signaling, mRNA levels of Id-1, the target gene of BMP-receptor signaling, were examined. As shown in Fig. 3B, treatment with BMP-4 (1 ng/ml) significantly upregulated Id-1 mRNA for 24 h, and the expression was significantly inhibited by co-treatment with orexin A (100 nM). Of note, treatment with the dual orexin receptor antagonist DORA-12 (10 µM) and the
selective OX1R antagonist SB408124 (10 \( \mu \)M) restored the suppressive effect of orexin on Id-1 mRNA expression induced by BMP-4 (Fig. 3B), suggesting that OX1R action is functionally linked to the suppression of BMP-Smad signaling in GH3 cells. To determine the role of OX1R function in GH3 cells, changes of OX1R mRNA in the presence of BMP-4 were also examined. As shown in Fig. 3C, addition of BMP-4 (10 ng/ml) downregulated OX1R mRNA expression for 24 h, suggesting that there is a counter-regulatory effect of BMP-4 for controlling PRL level via OX1R action.

To try to determine the underlying mechanism by which orexin A inhibits BMP-receptor signaling, the expression levels of BMP-receptor components were also examined by real-time PCR. As shown in Fig. 4A, cell culture with orexin A (100 nM) for 24 h reduced the expression levels of ALK-3 among the BMP-4 receptors expressed in GH3 cells, including ALK-2, ALK-3 and type II receptor BMPRII [26]. We next examined the effects of orexin A on the expression of inhibitory Smads. Since it is known that the expression of inhibitory Smad6/7 can be induced by stimulation of BMPs in various BMP-responsive cells [29-32], Smad6/7 mRNA levels were evaluated in GH3 cells in the presence of BMP-4 (10 ng/ml). As a result, treatment with orexin A (100 nM) significantly enhanced the mRNA expression of inhibitory Smad6 and Smad7 induced by BMP-4 (10 ng/ml) for 24 h (Fig. 4B). These findings suggested that OX1R signaling plays an inhibitory role in BMP-Smad signaling in GH3 cells via downregulation of BMP receptors and upregulation of inhibitory Smad6/7.

**Discussion**
Here we demonstrated a functional interaction between orexin A and BMP-4 in PRL secretion by pituitary lactotrope GH3 cells (Fig. 5). OX1R was expressed more dominantly than OX2R in GH3 cells, and OX1R activation induced by orexin A suppressed FSK- and BMP-4-induced PRL mRNA expression. Since orexin A treatment suppressed BMP-4-induced cAMP synthesis but not FSK-induced cAMP synthesis, the mechanism by which orexin A suppressed FSK-induced PRL expression is directly linked to BMP activity rather than the cAMP-PKA pathway in GH3 cells. Given that ALK inhibitors suppressed PRL expression, endogenous BMP action seems to upregulate PRL synthesis in an autocrine manner. Orexin A suppresses endogenous BMP activity in GH3 cells, leading to a reduction of FSK-induced PRL expression. It was of interest that orexin A treatment suppressed BMP-4-induced Smad1/5/9 signaling and Id-1 transcription via OX1R. It was also revealed that BMP signaling in GH3 cells was modulated by orexin A through suppression of BMP type-I receptor expression as well as upregulation of inhibitory Smad6/7. Treatment with BMP-4, in turn, suppressed OX1R expression, implying the presence of regulatory interaction of orexin and BMP-signaling in GH3 cells. (Fig. 5).

OX1R mRNA is expressed at a higher level than that of OX2R in the rat pituitary, and OX1R mRNA levels were significantly higher in male rats than in female rats [33]. Both OX1R mRNA and OX2R mRNA were expressed abundantly in the intermediate lobe in the rat pituitary, whereas OX1R was expressed more strongly than OX2R in the anterior lobe [9]. Immunofluorescence
analysis revealed that OX1R was present in acidophil cells that co-express GH, while OX2R was present in basophil cells that co-express ACTH [11]. In addition, in the Xenopus pituitary, OX1R was shown to be distributed in PRL-containing cells [34]. However, the physiological effect of orexin on PRL secretion in pituitary cells has remained controversial. In rat pituitary primary culture cells, orexin A did not affect PRL secretion [35], whereas Molik et al. found by using ovine cultured pituitary cells that PRL secretion in response to orexin A changed depending on the length of day [36]. Also, sheep PRL secretion was negatively responsive to orexin A during a short day, whereas orexin enhanced PRL secretion during a long day [36]. Given that the circadian modulator melatonin and Clock gene were shown to be linked to the regulation of PRL secretion via the function of BMP-4 [20], a new interaction between BMP-4 and orexin signaling might also be involved in control of the circadian profile of PRL secretion.

BMPs were originally recognized as factors for bone formation, but many physiological actions of BMPs in various endocrine tissues, including the ovary, pituitary, adrenal and thyroid, have been revealed [37, 38]. We earlier reported that rat lactotrope GH3 cells express BMP ligands including BMP-4 and -6, BMP type I (ALK-2, -3 and -4) and type II (ActRII, ActRIIB and BMPRII) receptors, and Smads (Smad1 to 8) [26]. We previously found that the BMP system is involved in PRL regulation by somatostatin analogs [19]. In addition, we reported that melatonin, related to the formation of circadian and seasonal rhythms, suppressed PRL production by inhibiting Smad signaling and cAMP synthesis [21]. BMPs have also been shown to be linked to follicle-stimulating hormone
(FSH) and luteinizing hormone secretion in gonadotropes cells [31, 39-42] and to the regulation of adrenocorticotropicin produced by corticotrope cells [16, 43].

Orexins were originally identified as neuropeptides in the hypothalamus, and attention has been paid to the localization where the orexin ligands can be produced and supplied for the pituitary. Preproorexin mRNA was not detected in the rat pituitary, as previously reported [33]; however, orexin nerve fibers and orexin ligands were abundantly expressed in the rat median eminence and posterior lobe of the pituitary [9]. Immunofluorescence analyses demonstrated the presence of both orexin A and orexin B in the human pituitary, wherein orexin A was distributed diffusely in the anterior pituitary and detected in more than 80% of lactotrope cells, and orexin B was found in all corticotrope cells. [44]. The existence of a low concentration of orexin A in human plasma has also been reported, and the levels apt to be affected by alteration of the energy status and body composition [4]. These results indicate the existence of circulating orexin from the hypothalamus via the pituitary portal artery or other peripheral tissues, although the source of orexin ligands remains unknown.

The present study demonstrated a physiological interaction of the orexin system and BMP signaling in lactotrope cells. Expression profiling analysis indicated that BMP-Smad signaling is one of the pathways regulated by orexin signaling [45]. In this regard, a novel effect of orexin on steroidogenesis of rat granulosa cells was uncovered by our recent approach [32]. It was found in that study that orexin A suppressed Smad-signaling by suppressing BMP receptors and by upregulating Smad6/7, which led to enhancement of FSH-induced progesterone synthesis [32]. Those findings are similar to the results of the
present study, implying that a functional link between the effects of orexin and BMPR signaling plays a key role in regulating hormonal homeostasis in various tissues. Considering that orexin A exerted inhibitory effects on BMP-4-induced PRL expression, orexin might also contribute to the formation of diurnal rhythm involving PRL secretion through regulation of BMP-4 activity in an in vivo situation. In the present study, a putative signaling of OX1R was indicated to be the P38-MAPK pathway based on the results of chemical inhibition experiments. However, the detailed mechanism by which OX1R activation suppresses BMP-4-induced PRL expression remains uncertain, and other pathways including PLC, PKC and intracellular Ca++ induction could also be involved in this activity [3].

Collectively, the results of the present study suggest that orexin A has an inhibitory role in PRL synthesis in the presence of PRL secretory factors. This may also be associated with circadian secretion of PRL. The results suggested that orexin plays a functional role as a modulator for BMP-4 activity that can facilitate PRL secretion (Fig. 5). From a clinical viewpoint, the findings may be applicable to treatment of prolactinomas, the most frequent functioning pituitary adenomas. Although dopamine agonists are widely and effectively used to treat most prolactinomas, some cases are resistant to treatment and some may recur [46]. Control of the endogenous BMP system that induces PRL secretion and an attempt to upregulate endogenous orexin activity might be a possible strategy for controlling prolactinomas.
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Figure Legends:

**Fig. 1. Expression of orexin receptors and effect of orexin A on PRL expression induced by FSK in GH3 cells.**  A) The expression of mRNAs encoding OX1R, OX2R, BMP-4 and RPL19 was examined by RT-PCR in GH3 cells compared with rat whole pituitary tissues. MM: molecular weight marker. B) After preculture, GH3 cells (1 × 10^5 cells/well) were treated with forskolin (FSK) and orexin A (ORX) in serum-free media. After 24-h culture, total cellular RNA was extracted and the mRNA expression levels of PRL were quantified by qPCR. The expression levels of target genes were standardized by the RPL19 level in each sample. C) After preculture, cells (5 × 10^4 cells/well) were treated with the combination of ORX and FSK in serum-free media containing 0.1 mM of IBMX. After 24-h culture, the supernatants of culture media were collected and cAMP levels were determined using ELISA. Results are shown as means ± SEM of data from at least three independent experiments with triplicated samples (B: n=9; C: n=6). Statistical analysis was performed by ANOVA. P values <0.05 were accepted as statistically significant. Values with different superscript letters are significantly different at P < 0.05.

**Fig. 2. Effects of orexin A on BMP-4-induced PRL expression and involvement of endogenous BMP action in GH3 cells.**  A, C, D) After preculture, GH3 cells (1 × 10^5 cells/well) were treated with ORX, BMP-4, FSK, and various signal inhibitors, LDN193189 (LDN), dorsomorphin (Dor), U0126, SB203580, SP600125 and H-89, in serum-free media. After 24-h culture, total
cellular RNA was extracted and the mRNA expression levels of PRL were quantified by qPCR. The expression levels of target genes were standardized by the RPL19 level in each sample. B) After preculture, cells (5 × 10^4 cells/well) were treated with ORX and BMP-4 in serum-free media containing 0.1 mM of IBMX. After 24-h culture, supernatants of the culture media were collected, and cAMP levels were determined using ELISA. Results are shown as means ± SEM of data from at least three independent experiments with triplicated samples (A: n=14; B: n=6; C: n=9; D: n=15). Statistical analysis was performed by ANOVA (A, B, D) and unpaired t-test (C). P values <0.05 were accepted as statistically significant. Values with different superscript letters are significantly different at P < 0.05. **P < 0.01 and *P < 0.05 vs. corresponding control groups, and #P < 0.05 vs. BMP-4-treated group.

**Fig. 3. Effects of orexin A on BMP-receptor signaling in GH3 cells.** A) GH3 cells (1 × 10^5 cells/well) were pretreated with ORX in serum-free media for 24 h. After 2-h stimulation with BMP-4, the cell lysates were subjected to immunoblot (IB) analysis using antibodies that detect pSmad1/5/9, tSmad1 and actin. B, C) Cells (1 × 10^5 cells/well) were treated with BMP-4, ORX, a dual ORX receptor antagonist (DORA-12), and an OX1R-selective antagonist (SB408124) in serum-free conditions for 24 h. Total cellular RNAs were extracted and the mRNA levels of Id-1 and OX1R were examined by qPCR. The expression levels of target genes were standardized by the RPL19 level in each sample. Results are shown as means ± SEM of data from at least three independent experiments with triplicated samples (A: n=7; B: n=15; C: n=11). Statistical analysis was
performed by ANOVA (A, B) and unpaired t-test (C). P values <0.05 were accepted as statistically significant. Values with different superscript letters are significantly different at P < 0.05. *P < 0.05 vs. between the indicated groups.

**Fig. 4. Effects of orexin A on the expression of genes involved in BMP-receptor signaling in GH3 cells.** GH3 cells (1 × 10⁵ cells/well) were treated with ORX (A) and the combination of ORX and BMP-4 (B). After 24-h culture, total cellular RNAs were extracted and the mRNA levels of ALK-2, ALK-3, BMPRII (A), Smad6 and Smad7 (B) were examined by qPCR. The expression levels of target genes were standardized by the RPL19 level in each sample. Results are shown as means ± SEM of data from at least three independent experiments with triplicated samples (A: n=12; B: n=15). Statistical analysis was performed by the unpaired t-test (A) and ANOVA (B). P values <0.05 were accepted as statistically significant. *P < 0.05 vs. between the indicated groups. Values with different superscript letters are significantly different at P < 0.05.

**Fig. 5. Functional interrelationship between orexin A and BMP-4 activities in pituitary lactotrope cells.** Orexin A inhibited BMP-4-induced PRL expression via OX1R on GH3 cells. Orexin A also suppressed FSK-induced PRL expression by inhibiting endogenous BMP action. Orexin A inhibited BMP-4-induced Smad1/5/9 phosphorylation and Id-1 transcription by downregulating ALK-3 and by upregulating BMP-4-induced Smad6/7, while BMP-4 suppressed OX1R expression. Thus, orexin A plays an inhibitory role in PRL production.
through suppression of BMPR signaling including endogenous BMP-4 action.

AC, adenylyl cyclase; BMPRs, BMP receptors; FSK, forskolin.