Original Article

Interaction between orexin A and bone morphogenetic protein system on progesterone biosynthesis by rat granulosa cells.

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Abbreviations:

AC, adenylate cyclase
ALK, activin receptor-like kinase
ActRII, activin type-II receptor
BMP, bone morphogenetic protein
BMPRII, BMP type-II receptor
DOR, dorsomorphin
FSH, follicle-stimulating hormone
FSHR, FSH receptor
GDF, growth and differentiation factor
GnRH, gonadotropin-releasing hormone
HPO, hypothalamic-pituitary-ovarian
3βHSD, 3β-hydroxysteroid dehydrogenase
LDN, LDN193189
ORX, orexin A
OX1 and OX2, orexin receptor type 1 and type 2
P450arom, P450 aromatase
P450scc, P450 steroid side-chain cleavage enzyme
StAR, steroidogenic acute regulatory protein

Abstract

The involvement of orexins in reproductive function has been gradually uncovered. However, the functional role of orexins in ovarian steroidogenesis remains unclear. In the present study, we investigated the effects of orexin A on ovarian steroidogenesis by using rat primary granulosa cells that express both OX1 and OX2 receptors for orexins. Treatment with orexin A enhanced progesterone, but not estradiol, biosynthesis induced by FSH, whereas it did not affect basal levels of progesterone or estradiol. In accordance with the effects on steroidogenesis, orexin A increased the mRNA levels of progesterogenic enzymes, including StAR, P450scc and 3βHSD, but not P450arom, and cellular cAMP synthesis induced by FSH. Under the condition of blockage of endogenous BMP actions by noggin or BMP-signaling inhibitors, orexin A failed to increase levels of progesterone synthesis induced by FSH treatment, suggesting that endogenous BMP activity in granulosa cells might be involved in the enhancement of progesterone synthesis by orexin A. Treatment with orexin A impaired Smad1/5/9 activation as well as Id-1 mRNA expression stimulated by

BMP-6 and BMP-7, the latter of which was reversed by treatment with an OX1 antagonist. It was also found that orexin A suppressed the mRNA expression of both type-I and -II receptors for BMPs and increased that of inhibitory Smad6 and Smad7 in granulosa cells. On the other hand, treatments with BMP-6 and -7 suppressed the expression of OX1 and OX2. Collectively, the results indicated that orexin A enhances FSH-induced progesterone production, at least in part, by downregulating BMP signaling in granulosa cells. Thus, a new role of orexin A in facilitating progesterone synthesis and functional interaction between the orexin and BMP systems in granulosa cells were revealed (276 words).

Introduction

Orexins A and B are neuropeptides that are mainly synthesized in the hypothalamus in the process of proteolytic cleavage from the common precursor prepro-orexin [1, 2]. Orexins play key roles in the control of sleep-wakefulness, energy balance and food intake. The actions of orexins are mediated via two orexin receptors, OX1 and OX2: OX1 is selective for orexin A and OX2 binds both orexin A and orexin B [3]. It has also been shown that orexins and their receptors are expressed in various peripheral tissues outside the central nervous system [4]. Recently, the existence of an interrelationship between the orexinergic and reproductive systems, including the actions of orexins on hypothalamic gonadotropin-releasing hormone (GnRH) neurons and pituitary gonadotropes, has been recognized [5, 6].

As for the action of orexins in the hypothalamic-pituitary-ovarian (HPO) axis, Silveyra *et al.* [7] reported that orexin receptors were expressed in the brain and pituitary of female cycling rats and that the expression levels of OX1, OX2

and prepro-orexin were increased in the hypothalamus and pituitary during the proestrus evening. Nitkiewicz *et al.* [8] also confirmed the expression of OX1 and OX2 in porcine ovaries at the gene and protein levels and showed that the levels of OX1 and OX2 expression changed according to the cycle. The cycle-dependent expression of orexin receptors suggested physiological significance of the ovarian orexin system. The finding that orexin ligands exist in all follicular stages of adult ovaries of other species such as cats and dogs indicated that the ovary might be a major potential site of orexinergic activity [9].

Regarding ovarian steroidogensis in relation to orexin, Cataldi *et al.* [10] found that the expression levels of OX1 and OX2 were increased and the production of progesterone was reduced by treatment of luteal cells from superovulated rat ovaries with orexins. Inhibitory effects of orexin on estradiol synthesis induced by follicle-stimulating hormone (FSH) in porcine granulosa cells also suggested a regulatory role of orexins in reproductive functions through modulation of ovarian steroidogenesis [11].

On the other hand, recent studies have demonstrated that ovarian growth factors play critical roles in female fertility in mammals in an autocrine/paracrine manner [12]. Complex interactions between gonadotropins and ovarian autocrine/paracrine factors including activins/inhibins, bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs) are critical for follicle growth and maturation. BMP system molecules consisting of the units of BMP ligands and receptors are cell-specifically expressed in ovarian follicles. The ovarian BMP system regulates FSH receptor activity in granulosa cells, leading to a critical control of ovarian folliculogenesis in an autocrine/paracrine manner [12-14]

In the present study, we used a primary culture of rat granulosa cells to investigate the functional roles of orexins, particularly in steroidogenesis induced by FSH with focus on the ovarian BMP system. The results of experimental studies suggested the involvement of orexins at various points of the reproductive axis; however, the impact of orexins on steroidogenetic cascades has yet to be determined. The present study uncovered a novel activity of orexins in

progesterone biosynthesis and its functional interaction with the ovarian BMP system in granulosa cells.

Materials and Methods

Experimental reagents and supplies

Culture media including HEPES buffer solution, McCoy's 5A and Medium 199 were purchased from Invitrogen Corp. (Carlsbad, CA). Hormones and chemicals including 4-androstene-3,17-dione, diethylstilbestrol (DES), 3-isobutyl-1-methylxanthine (IBMX), ovine pituitary FSH, and penicillin-streptomycin solution were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Recombinant proteins including human BMP-6 and BMP-7 and mouse Noggin were obtained from R&D Systems Inc. (Minneapolis, MN), and human Orexin A was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). BMP-receptor signaling inhibitors, LDN193189 and dorsomorphin, were from Stemgent (San Diego, CA) and Calbiochem (San Diego, CA), respectively. A selective

non-peptide OX1 antagonist, SB408124 [15], was purchased from Tocris Bioscience (Bristol, UK).

Procedure for primary culture of granulosa cells

Silastic capsules containing 10 mg of DES powder were implanted subcutaneously in 22-day-old female Sprague-Dawley rats (purchased from Charles River, Wilmington, MA). After 3- to 4-day exposure to DES, granulosa cells were obtained by puncturing ovarian follicles with a 27-gauge needle. The granulosa cells were counted and separated from oocytes by filtering the cell suspension through serial nylon meshes of 100 and 40 μm in size (BD Falcon, Bedford, MA), which allowed granulosa cells but not oocytes to pass through [16, 17]. Subsequently, the isolated granulosa cells were cultured in a serum-free McCoy's 5A medium supplemented with penicillin-streptomycin at 37°C in an atmosphere of 5% CO_2. A range of orexin A concentrations from 1 nM to 300 nM was screened on the basis of results of earlier *in vitro* studies [10, 11], and a concentration of 100 nM was shown by preliminary experiments to exert

significant changes in ovarian steroidogenesis. The concentrations of FSH (10 ng/ml), BMPs (100 ng/ml), noggin (30 ng/ml), dorsomorphin and LDN193189 (300 nM) were chosen on the basis of results of our previous experiments using the same granulosa cell culture conditions [18-20]. The present animal protocols (OKU-2016065) were approved by Okayama University Institutional Animal Care and Use Committee.

Measurements of the concentrations of steroids and cAMP

Rat granulosa cells (1×10^5 viable cells in 200 µl) were cultured, as described above, in 96-well plates with androstenedione (100 nM), a substrate for aromatase. FSH (10 ng/ml) was added to the culture medium either alone or in combination with the indicated concentrations of orexin A, noggin, dorsomorphin and LDN193189 for 48 h. The concentrations of estradiol and progesterone in the collected culture media were examined by a chemiluminescent immunoassay (CLIA) using Architect estradiol and progesterone kits (Cayman Chemical Co., Ann Arbor, MI, USA). In a cell-free medium, steroid contents were undetectable

(progesterone content of <10 pg/ml and estradiol content of <15 pg/ml). To evaluate cellular synthesis of cAMP, rat granulosa cells (1×10^5 viable cells in 200 µl) were cultured, as described above, in 96-well plates for 48 h in the presence of 0.1 mM IBMX, which is a specific inhibitor of phosphodiesterase activity. The levels of extracellular cAMP in the collected culture media were determined by an enzyme-linked immunosorbent assay (ELISA; Enzo Life Sciences, Inc., NY, USA) after sample acetylation with an assay sensitivity of 0.039 pmol/ml.

Cellular RNA extraction and quantitative RT-PCR

Rat granulosa cells (5×10^5 viable cells in 1 ml) were cultured in 12-well plates with serum-free McCoy's 5A medium. The cells were treated with FSH (10 ng/ml) either alone or in combination with the indicated concentrations of orexin A, BMP-6, BMP-7 and SB408124. After 24-h or 48-h culture, total cellular RNA was extracted using TRI Reagent® (Cosmo Bio Co., Ltd., Tokyo, Japan). Total RNA amount was quantified by using a NanoDrop™ One spectrophotometer

(Thermo Fisher Scientific, Waltham, MA), and the RNA was stored at -80°C until assay. Primer pairs for *Acvr1* (also known as activin receptor-like kinase; ALK-2), *Bmpr1a* (ALK-3), *Acvr2a* (activin type-II receptor, ActRIIA), *Smad6* and *Smad7*, and ribosomal protein L19, *Rpl19* were selected as reported previously [21, 22]. For all of the genes, the primer pairs were selected from different exons of the corresponding genes to distinguish PCR products that might arise from chromosome DNA contaminants as follows: *Cyp11a* (P450scc), 147-167 and 636-655 (J05156); steroidogenic acute regulatory protein, *Star*, 395-415 and 703-723 (AB001349); type-I 3β-hydroxysteroid dehydrogenase, *Hsd3b* (3βHSD), 336-355 and 841-860 (M38178); *Cyp19* (P450arom), 1180-1200 and 1461-1481 (M33986); *Id1*, 225-247 and 364-384 (NM_010495); *Bmpr1b* (ALK-6), 227-246 and 450-469 (NM_001024259); BMP type-II receptor, *Bmpr2* (BMPRII), 1785-1804 and 1942-1961 (NM_080407); orexin receptor type 1, *Hctr1* (OX1), 1658-1680 and 2057-2079 (NM_013064); and orexin receptor type 2, *Hctr2* (OX2), 209-233 and 539-558 (NM_013074). The amplification efficiency of each primer was as follows: *Rpl19*, 92%; *Star*, 94%; *Cyp11a*, 96%; *Hsd3b*, 97%; *Cyp19*, 96%;

Id1, 91%; *Actvr1*, 99%; *Bmpr1a*, 86%; *Bmpr1b* 87%; *Bmpr2*, 96%; *Actvr2a*, 98%, *Smad6*, 96%; *Smad7*, 86%; *Hctr1*, 91%; *Hctr2*, 90%; and *Fshr*, 96%. One μg of the extracted RNA was used for reverse transcription reaction by a ReverTra Ace® (TOYOBO CO., LTD., Osaka, Japan) with random hexamer and deoxynucleotide triphosphate (dNTP) at 42°C for 50 min and at 70°C for 10 min. Quantitative PCR was performed to quantify the level of target gene mRNA using the LightCycler® Nano real-time PCR system (Roche Diagnostic Co., Tokyo, Japan) after optimizing the annealing conditions. Relative mRNA expression was calculated by using the Δ threshold cycle (Ct) method. The values of ΔCt were obtained by subtracting the Ct value of *Rpl19* mRNA from that of the target mRNA. The target mRNA level relative to *Rpl19* mRNA was expressed as $2^{-(\Delta Ct)}$ and the results were expressed as the ratios of target to RPL19 mRNA.

Cell lysate preparation and Western blots

Rat granulosa cells (2.5×10^5 viable cells in 500 μl) were cultured in 24-well plates in serum-free McCoy's 5A medium. After preculture either alone or with

the indicated concentrations of orexin A for 24 h, the cells were stimulated with BMP-6 and -7 for 60 min. The treated cells were solubilized in 50 μl RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY) containing 1 mM Na_3VO_4, 1 mM NaF, 2% SDS, and 4% β-mercaptoethanol. SDS-PAGE/immunoblotting analysis was then performed using the cell lysates with antibodies against phospho-Smad1/5/9 (pSmad1/5/9), total-Smad1 (tSmad1; Cell Signaling Technology, Inc., Beverly, MA) and actin (Sigma-Aldrich Co. Ltd.). Each band was analyzed by the C-DiGit® Blot Scanner System (LI-COR Biosciences, NE) as the integrated signal density. Ratios of the signal intensities of pSmad/tSmad were calculated for evaluating the target protein contents.

Statistical analysis

Results are shown as means ± SEM of data from at least three independent experiments with triplicated samples. Statistical analysis was performed by ANOVA and the unpaired *t*-test (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). When significant differences were detected by ANOVA, Tukey-

Kramer's post hoc test was subsequently performed (StatView 5.0 software). *P* values <0.05 were accepted as statistically significant.

Results

First of all, we examined the expression of orexin receptors in rat granulosa cells. As shown in **Fig. 1A**, the expression of both type 1 and type 2 receptors, *Hctr1* and *Hctr2*, was detected by RT-PCR. The effects of orexin A on FSH-induced ovarian steroidogenesis were examined by using rat primary granulosa cells cultured for 48 h. Treatment with orexin A (100 nM) alone had no significant effect on basal levels of progesterone and estradiol (**Fig. 1B**) synthesis. Of note, treatment with orexin A (100 nM) significantly increased progesterone synthesis induced by FSH (10 ng/ml). In contrast, orexin A (100 nM) had no significant effect on FSH-induced estrogen synthesis by rat granulosa cells (**Fig. 1B**).

Given that the ovarian BMP system plays a regulatory role in FSH-induced progesterone synthesis but not estradiol synthesis, it was assumed that

endogenous BMP activity was involved in the orexin effects on FSH-induced steroidogenesis by granulosa cells. We tried to block the endogenous BMP action by extracellular and intracellular approaches. Of note, under the condition of co-culture with the BMP-binding protein noggin (30 ng/ml), orexin A (100 nM) treatment failed to increase FSH-induced progesterone production by rat granulosa cells (**Fig. 1C**). Compounds of dorsomorphin, which selectively inhibits the signaling induced by ALK-2, -3 and -6 [23], and LDN193189, which also blocks ALK-2 and -3 actions with higher activity and specificity [24], were utilized to inhibit endogenous BMP-receptor activity. As shown in **Fig. 1C**, orexin A failed to increase FSH-induced progesterone production in the presence of dorsomorphin or LDN193189 as was seen in the case of noggin, indicating that endogenous BMP action is necessary to enhance FSH-induced steroidogenesis by orexin A.

To clarify the mechanism by which orexin A increases FSH (10 ng/ml)-induced steroidogenesis by granulosa cells, the change in cAMP synthesis was determined by ELISA. As shown in **Fig. 1D**, FSH-induced cAMP production was

increased by treatment with orexin A (100 nM) for 48 h, although basal levels of cAMP synthesis were not affected by orexin A. In accordance with the effects of orexin A on steroid biosynthesis, orexin A (100 nM) did not affect basal expression of steroidogenic factor and enzyme mRNAs, but it was found to enhance mRNA levels of progesterogenic factors and enzymes including *Star*, *Cyp11a* and *Hsd3b* stimulated by FSH (10 ng/ml) (**Fig. 1E**). On the other hand, the level of FSH (10 ng/ml)-induced *Cyp19* mRNA was not altered by treatment with orexin A (100 nM) (**Fig. 1E**). In addition, as shown in **Fig. 1F**, *Fshr* expression was not affected by treatment with orexin A.

To elucidate the effects of orexin A on BMP-receptor signaling in granulosa cells, Smad1/5/9 phosphorylation and transcription of the BMP target gene Id-1 induced by BMP-6 and -7 were examined in the presence of orexin A. As shown in **Fig. 2A**, stimulation with BMP-6 and -7 (100 ng/ml) readily induced Smad1/5/9 phosphorylation for 1 h, although orexin A (100 nM) alone had no effect on Smad1/5/9 phosphorylation. Of note, co-treatment with orexin A (100 nM) for 24 h significantly suppressed the Smad1/5/9 phosphorylation induced by

BMP-6 and -7 (100 ng/ml) (**Fig. 2A**). In accordance with the effects on Smad activation, levels of *Id1* mRNA induced by BMP-6 and BMP-7 (100 ng/ml) were also suppressed by co-treatment with orexin A (100 nM) for 24 h (**Fig. 2B**). In this experiment, SB408124, a non-peptide antagonist that is relatively selective for OX1, was also used to examine whether orexin action can act via OX1 receptors. The inhibitory effects of orexin A on *Id1* expression induced by BMP-6 and -7 were reversed in the presence of an OX1 antagonist (**Fig. 2B**), suggesting that orexin A action to inhibit BMPR signaling can occur, at least in part, through the activity OX1.

In order to assess the mechanism by which orexin A suppresses BMP-receptor signaling, the expression of each BMP-receptor component was examined by quantitative PCR. As shown in **Fig. 3A**, treatment with orexin A (100 nM) for 48 h decreased mRNA expression of BMP type-I receptors (*Acvr1*, *Bmpr1a* and *Bmpr1b*) and that of type-II receptors (*Acvr2a* and *Bmpr2*). In addition, orexin A (100 nM) increased the mRNA expression of inhibitory *Smad6* and *Smad7* in the presence of BMP-6 and -7 (100 ng/ml) for 24 h (**Fig. 3B**). On

the other hand, treatment with BMP-6 and -7 (100 ng/ml) for 48 h significantly suppressed the mRNA expression of orexin receptors including *Hctr1* and *Hctr2* (**Fig. 3C**), suggesting the existence of a functional feedback between the BMP and orexin systems in rat granulosa cells.

Discussion

The involvement of orexin activity at different levels in the HPO axis has been recognized. The central actions of orexins on the reproductive axis consist of hypothalamic GnRH induction from the hypothalamus and subsequent or direct luteinizing hormone secretion from the pituitary [5]. Regarding the orexinergic system in the ovary, it was shown that OX1 and OX2 were expressed at distinct growing stages during the estrous cycle and that their expression was correlated with hormonal circumstances [7]. A study on the porcine ovary regarding stage-dependent gene expression of prepro-orexin and immunoreactivity of orexins indicated that the pattern of orexin expression is linked to hormonal conditions by modulating ovarian steroidogenesis [11]. Thus, the orexin system in the ovary is mutually linked to changes in the hormonal environment related to female reproduction.

In the present study, it was revealed that orexin A treatment amplifies progesterone synthesis but not estrogen synthesis induced by FSH in rat granulosa cells expressing both orexin receptors OX1 and OX2 (**Fig. 4**). This

finding was also confirmed by steroidogenic enzyme expression and cellular cAMP synthesis induced by FSH. It was of interest that neutralization of endogenous BMPs by noggin, which extracellularly inhibits the effects of BMP-2, -4, -5, -6 and -7 and GDFs [25], and BMPR-signal inhibitors, such as dorsomorphin and LDN193189, abolished the orexin effects on progesterone production caused by FSH, indicating functional involvement of the endogenous BMP system in the effects of orexin A on progesterone synthesis. Furthermore, it was revealed that BMP signaling in granulosa cells was impaired by orexin A through suppression of BMP type-I and -II receptor expression and upregulation of inhibitory Smad6 and 7 expression. On the other hand, treatments with BMP-6 and -7 suppressed the receptor expression of *Hctr1* and *Hctr2*, implying the presence of mutual modulation between BMP and orexin activities in granulosa cells (**Fig. 4**).

The existence of functional regulation of female fertility by TGF-β superfamily members, including activins, inhibins and BMPs/GDFs, has been established [12, 26, 27]. BMP system molecules including various BMP ligands,

their receptors and binding proteins are expressed in follicular cells in a cell-dependent fashion, and their expression can be dynamically altered depending on the developmental phases of ovarian follicles [12]. Among the BMP ligands affected by binding to noggin, BMP-2 and -4 can bind to type-I receptors of ALK-3/ALK-6, and BMP-6 or -7 has a higher affinity to ALK-2/ALK-6 [28-31], while BMP-15 has a higher affinity to ALK-6 than to ALK-3 [32]. Regarding the BMP type-II receptors, BMPRII has exclusive binding capacity to major ovarian BMP ligands such as BMP-2, -4, -6, -7 and -15 [32-35], while ActRII binds to BMP-6 and -7 [29, 30]. In the present study, it was found that orexin A worked to downregulate cellular Smad1/5/9 signaling elicited by BMP-6 and -7 through suppression of type-I and -II receptor expression and inhibitory Smads6/7 expression in granulosa cells. Taking into account the fact that the genes regulated by orexin receptor signaling are functionally associated with cellular growth and metabolism [3, 36], such an elaborate interaction between BMP-to-Smad signaling and orexin-receptor signaling might be linked to the fine-tuning of ovarian steroidogenesis in growing follicles.

Experiments on blocking of orexin receptors also demonstrated a significant impact on the ovary [37]. Treatments with orexin-receptor antagonists reduced the number of released ova in the oviduct and increased the number of preovulatory follicles but decreased the number of corpora lutea, implying a certain defect in the ovulation process [37]. On the basis of the present results, one of the major effects of orexin A is suppression of BMP receptor signaling in the ovary. Namely, blocking of orexin receptors fails to suppress endogenous BMP actions, leading to a lack of luteinizing inhibition, which may cause ovulation failure. In the present study, inhibitory effects of orexin A on *Id1* expression induced by BMP-6 and -7 were reversed in the presence of an OX1 antagonist, suggesting that orexin A action to inhibit BMP-receptor signaling can occur through the OX1 receptor. Considering the functional role of the BMP system that inhibits luteinization and progesterone synthesis by suppressing FSH responsiveness in granulosa cells [12, 27, 38, 39], it is possible that orexin A actions in the ovary, at least in part, can be elicited through the inhibition of endogenous BMP activity.

As for the possible source of orexin ligands for ovarian receptors, the presence of preproorexin transcripts in porcine ovaries [11] suggests the existence of a local auto/paracrine activity of orexin, but the absence of preproorexin expression in rat ovaries [37, 40] indicates species-dependent differences in the source of orexin ligands. It is thus presumed that orexins are transferred from the circulating pool or that receptor actions are elicited by some mechanisms other than orexin ligands. Although the origin of circulating orexin has not been determined, the concentrations tend to be influenced by the alteration of energy balance or metabolic homeostasis [4]. In any case, further studies are required to determine the source of orexin ligands and their local role in the ovarian orexin system.

Collectively, the results indicate that orexin A enhances FSH-induced progesterone production, at least in part, by downregulating BMP signaling in rat granulosa cells, while BMPs suppress the expression of orexin receptors (**Fig. 4**). The inhibition of endogenous BMP signaling by orexin A leads to enhancement of FSH-induced progesterone levels; however, the abrogation of endogenous

BMP activity seems likely to make granulosa cells less sensitive to orexin action. The detailed mechanism by which blockage of BMP-signaling inactivates orexin effects remains unclear in the present study. Nevertheless, a novel activity of orexin A in the upregulation of progesterone synthesis and its functional interaction with the ovarian BMP system were demonatrated in granulosa cells. These findings indicate the possibility that orexin may be applicable for induction of luteinization and/or maintenance of pregnancy.

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Figure legends:

Fig. 1. Expression of orexin receptors and effects of orexin A on FSH-induced steroidogenesis by rat granulosa cells. **A**) Total cellular RNA was extracted from granulosa cells, and the expression of *Hctr1* (422 bp), *Hctr2* (350 bp) and *Rpl19* (190 bp) was examined by RT-PCR. **B**) Granulosa cells were cultured in a serum-free condition with FSH either alone or in combination with orexin A (ORX). After 48-h culture, the levels of progesterone and estradiol in the medium were determined by CLIA, and the levels of progesterone and estradiol were expressed as fold changes. **C**) Granulosa cells were cultured in the presence of noggin (Nog), dorsomorphin (DOR) and LDN193189 (LDN) in a serum-free condition with FSH either alone or in combination with ORX. After 48-h culture, the levels of progesterone in the medium were determined by CLIA, and the levels of progesterone were expressed as fold changes. **D**) Granulosa cells were cultured in a serum-free medium containing IBMX with FSH either alone or in combination with ORX. After 48-h culture, the levels of cAMP in the medium were determined by EIA. **E**, **F**) Total cellular RNA was extracted from

granulosa cells treated with FSH and ORX for 48 h in a serum-free condition, and *Star*, *Cyp11a*, *HSD3b*, *Cyp19* and *Fshr* mRNA levels were determined by quantitative PCR. The expression levels of target gene mRNA were standardized by *Rpl19* level and expressed as fold changes. Results in all panels are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA. Values with different superscript letters are significantly different at $P < 0.05$. MM indicates molecular weight marker.

Fig. 2. Effects of orexin A on BMP-receptor signaling in rat granulosa cells.

A) After preculture in a serum-free condition with orexin A (ORX) for 24 h, granulosa cells were stimulated with BMP-6 and -7 for 60 min. The cells were lysed and subjected to SDS-PAGE/immunoblotting analysis using anti-pSmad1/5/9 and anti-actin antibodies. The signal intensities of pSmad1/5/9 were standardized by tSmad1 signal intensities in each sample and then expressed as fold changes. **B**) Total cellular RNA was extracted from granulosa

cells treated with BMP-6 and BMP-7 either alone or in combination with ORX and the OX1 antagonist SB408124 for 24 h, and *Id1* mRNA levels were determined by quantitative PCR. The expression levels of target gene mRNA were standardized by RPL19 level and expressed as fold changes. Results in all panels are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA. Values with different superscript letters are significantly different at $P < 0.05$.

Fig. 3. Effects of orexin A on the expression of BMP receptors and effects of BMPs on the expression of orexin receptors in rat granulosa cells. **A**) Total cellular RNA was extracted from granulosa cells treated with orexin A (ORX) for 48 h, and mRNA levels of BMP type-I and type-II receptors were determined by quantitative PCR. **B**) Total cellular RNA was extracted from granulosa cells treated with ORX in the presence of BMP-6 and -7 for 24 h, and mRNA levels of *Smad6* and *Smad7* were determined by quantitative PCR. **C**) Total cellular RNA

was extracted from granulosa cells treated with BMP-6 and -7 for 48 h, and mRNA levels of *Hctr1* and *Hctr2* were determined by quantitative PCR. The expression levels of target gene mRNA were standardized by *Rpl19* level and expressed as fold changes. Results in all panels are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by the unpaired *t*-test. $^{**}P < 0.01$ and $^{*}P < 0.05$ vs. control group.

Fig. 4. Possible mechanism by which orexin A modulates ovarian steroidogenesis in rat granulosa cells. Orexin A increases FSH-induced progesterone production but not estradiol production by granulosa cells. The orexin action on progesterone synthesis is blocked in the presence of the BMP-binding protein noggin (Nog) or the BMPR-signal inhibitor dorsomorphin (DOR) or LDN193189 (LDN). Orexin A suppresses BMP-6- and -7-induced Smad1/5/9 signaling by downregulating the expression of BMP type-I and type-II receptors and by upregulating inhibitory Smads6/7. BMPs downregulate the expression

of orexin receptors OX1 (*Hctr1*) and OX2 (*Hctr2*) on rat granulosa cells. Orexin A plays an enhancing role in progesterone production by downregulating BMP-receptor signaling that inhibits FSH-induced progesterone synthesis through suppression of cAMP synthesis via inhibition of adenylate cyclase (AC) in granulosa cells.