Original Article

Regulatory role of melatonin and BMP-4 in prolactin production by rat pituitary lactotrope GH3 cells.

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Abstract

The effects of melatonin on prolactin production and its regulatory mechanism remain uncertain. We investigated the regulatory role of melatonin in prolactin production using rat pituitary lactotrope GH3 cells by focusing on the bone morphogenetic protein (BMP) system. Melatonin receptor activation, induced by melatonin and its receptor agonist ramelteon, significantly suppressed basal and forskolin-induced prolactin secretion and prolactin mRNA expression in GH3 cells. The melatonin MT2 receptor was predominantly expressed in GH3 cells, and the inhibitory effects of melatonin on prolactin production were reversed by treatment with the receptor antagonist luzindole, suggesting functional involvement of MT2 action in the suppression of prolactin release. Melatonin receptor activation also suppressed BMP-4-induced prolactin expression by inhibiting phosphorylation of Smad and transcription of the BMP-target gene Id-1, while BMP-4 treatment upregulated MT2 expression. Melatonin receptor activation suppressed basal, BMP-4-induced and forskolin-induced cAMP synthesis; however, BtcAMP-induced prolactin mRNA expression was not affected by melatonin or ramelteon, suggesting that MT2 activation leads to inhibition of prolactin production through the suppression of Smad signaling and cAMP synthesis. Experiments using intracellular signal inhibitors revealed that the ERK pathway is, at least in part, involved in prolactin induction by GH3 cells. Thus, a new regulatory role of melatonin involving BMP-4 in prolactin secretion was uncovered in the pituitary lactotrope.

Introduction

Melatonin, a lipophilic indoleamine synthesized from serotonin by the pineal gland, is involved in the formation of physiological circadian and seasonal rhythms [1]. Melatonin acts mainly through MT1 and MT2 receptors, which are expressed in various tissues and cells centrally and peripherally [2]. Melatonin also plays a role as a scavenger for reactive oxygen-nitrogen species via receptor-unrelated mechanisms. In vertebrates, melatonin actions are linked not only to the circadian rhythm but also to the activities of hormones and cytokines [3, 4]. In the major endocrine axis, melatonin has been shown to suppress adrenocorticotropin (ACTH)-induced cortisol production in the adrenal [5, 6] and ACTH secretion from the anterior pituitary. In this regard, we recently reported that melatonin suppresses ACTH secretion, at least in part, through the effects of bone morphogenetic protein (BMP)-4, which is expressed in pituitary corticotrope cells [7].

BMPs are growth factors that belong to the transforming growth factor (TGF)-β superfamily. Various physiological BMP actions in endocrine tissues, including the ovary, pituitary, thyroid and adrenal, have been discovered [8, 9]. There is also increasing evidence that locally produced BMPs play key roles in differentiation of the pituitary. The BMP system is known to play important roles in initial development of the anterior pituitary [10]. BMP-4 is required during the first stage of pituitary organogenesis accompanying proliferation of Rathke's pouch, which gives rise to Pit-1 lineage cells including lactotrope cells. Of interest, BMP-4, a critical factor for pituitary organogenesis, also plays a key role in the

pathogenesis of differentiated pituitary lineages [11-14]. Overexpression of BMP-4 in various models of lactotrope tumors including dopamine D2-receptor null mice and estrogen-induced rat prolactinomas and also in human prolactinomas was shown for the first time by Arzt's group by demonstrating a crosstalk between BMP-4 signaling and the estrogen receptor (ER) for the possible pathogenesis of prolactinomas [15]. The same group also revealed the detailed molecular interaction encompassing BMP-4, Smads and ER that is functionally involved in the regulation of prolactin (PRL)-promoter activity [16].

In this regard, we previously reported that rat lactotrope GH3 cells express BMP/activin type I receptors including ALK-2, -3 and -4, type II receptors including ActRII, ActRIIB and BMPRII, and Smads including Smad1 to Smad8 [17]. We also revealed the involvement of the BMP system in regulation of PRL secretion controlled by somatostatin [18], in which BMP-4 and -6 have differential effects on the expression of somatostatin receptors in GH3 cells. BMPs are also functionally linked to the regulation of follicle-stimulating hormone and luteiniing hormone secretion by gonadotropes cells [19-23].

In the present study, we investigated the effects of melatonin with a focus on BMP-4 activity in the regulation of PRL secretion by pituitary lactotrope cells. The increase in melatonin at night plays a key role as a biological clock, which also affects the immune system, antioxidant actions, and secretion of various hormones and growth factors. However, the effect of melatonin on PRL secretion and the regulatory mechanism in the anterior pituitary has not been clarified. A functional link between melatonin and the BMP system for PRL production was newly uncovered.

Materials and Methods

Reagents and supplies

A 1:1 mixture of Dulbecco's Modified Eagle's Medium/Ham F-12 medium (DMEM/F12), penicillin-streptomycin solution, forskolin (FSK), melatonin, luzindole, N^6,O^2-dibutyryl adenosine-3',5'-cyclic monophosphate monosodium salt (BtcAMP), 3-isobutyl-1-methylxanthine (IBMX), and H-89 (cAMP-dependent protein kinase inhibitor) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). The MT1/MT2 agonist ramelteon [24] was provided by Takeda Pharmaceutical Co., Ltd. (Osaka, Japan). Recombinant human BMP-4 was purchased from R&D Systems Inc. (Minneapolis, MN), the ERK inhibitor U0126 and 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). Normal rat pituitary tissues were obtained from 8-week-old female Wistar rats.

Cell culture and cAMP measurement

Rat pituitary tumor GH3 cells were provided by Prof. Joseph A. Majzoub, Children's Hospital, Harvard Medical School. The cells were cultured in DMEM/F12 medium supplemented with 10% fetal calf serum (FCS) and antibiotics in a 5% CO_2 atmosphere at 37°C [17, 18]. GH3 cells (1×10^5 viable cells) were seeded in 96-well plates with DMEM/F12 containing 10% FCS and

penicillin-streptomycin. After preculture, the medium was changed to serum-free DMEM/F12 containing penicillin-streptomycin and 0.1 mM IBMX (a specific inhibitor of phosphodiesterase activity), and then the cells were treated with indicated concentrations and combinations of FSK, melatonin, ramelteon, and BMP-4. After 24-h culture, the medium was collected and centrifuged. The supernatant of the culture media was collected and stored at -80°C until assay. After acetylation of each sample, the extracellular contents of cAMP were determined by an enzyme immunoassay with assay sensitivity of 0.039 nM (Assay Designs, Inc., Ann Arbor, MI). The intra- and inter-assay coefficients of variation are 6.8% and 7.9%, respectively.

Determination of PRL levels

GH3 cells (1×10^5 viable cells) were cultured in 96-well plates with DMEM/F12 containing 10% FCS and penicillin-streptomycin. After preculture, the medium was changed to serum-free DMEM/F12, and then the cells were treated with indicated concentrations and combinations of FSK, melatonin, ramelteon, and BMP-4. After 24-h culture, the incubation medium was collected and centrifuged. The supernatant of the culture media was collected and stored at -80°C until assay. The levels of PRL in the cultured media were determined by a rat-specific enzyme immunoassay with assay sensitivity of 1 pg/ml [25] (SPI-BIO, Montigny-le-Bretonneux, France). The intra- and inter-assay coefficients of variation are 10.6% and 13.4%, respectively.

RNA extraction and quantitative real-time PCR analysis

After preculture, cells (3×10^5 viable cells) in 12-well plates were treated with indicated concentrations of FSK, BtcAMP, melatonin, ramelteon, BMP-4, and various signal inhibitors in serum-free DMEM/F12. After 24-h culture, the medium was removed and total cellular RNA was extracted using TRIzol® (Invitrogen Corp., Carlsbad, CA). Total RNA was quantified by measuring the absorbance of the sample at 260 nm and was stored at -80°C until assay. Oligonucleotides used for RT-PCR were custom-ordered from Invitrogen Corp. PCR primer pairs were selected from different exons of the corresponding genes as follows: PRL: 271-291 and 471-491 (NM_012629); Id-1: 218-240 and 357-377 (NM_012797); MT1 receptor: 350-370 and 651-671 (NM_053676); MT2 receptor: 678-697 and 882-901 (NM_001100641); and ribosomal protein L19 (RPL19): 401-421 and 575-595 (J02650). The extracted RNA (1 μg) was subjected to an RT reaction using a First-Strand cDNA Synthesis System (Invitrogen Corp.) with random hexamer (2 ng/μl), reverse transcriptase (200 U) and deoxynucleotide triphosphate (dNTP; 0.5 mM) at 42°C for 50 min and at 70°C for 10 min. Aliquots of PCR products were electrophoresed on 1.5% agarose gels and visualized after ethidium bromide staining. For the quantification of each target mRNA level, real-time PCR was performed using the Light Cycler® Nano real-time PCR system (Roche Diagnostics, Tokyo, Japan) under optimized annealing conditions according to the manufacturer's protocol with the following profile: 40 cycles each at 95°C for 3 sec and 60°C for 30 sec. The threshold cycle (Ct) values were calculated using the Light Cycler® Nano real-time PCR system software Ver1.0 (Roche Diagnostics). The relative expression of each mRNA was calculated by the ΔCt method, in which ΔCt is the value obtained by subtracting the Ct value of

Rpl19 mRNA from the Ct value of the target mRNA, and the amount of target mRNA relative to *Rpl19* mRNA was expressed as $2^{-(\Delta Ct)}$. The data are expressed as the ratio of target mRNA to *Rpl19* mRNA.

Western immunoblot analysis

Cells (3×10^5 viable cells) were cultured in 12-well plates in DMEM/F12 containing penicillin-streptomycin. After preculture, the medium was changed to serum-free DMEM/F12 and treated with indicated concentrations and combinations of FSK, melatonin, ramelteon and luzindole. After 1-h culture stimulation with BMP-4 or FSK, the cells were then solubilized by a sonicator 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. The cell lysates were then subjected to SDS-PAGE immunoblotting analysis using anti-phospho-Smad1/5/9 antibody, anti-phospho- and anti-total-ERK1/2 (pERK and tERK), P38 (pP38 and tP38) and SAPK/JNK (pJNK and tJNK) antibodies (Cell Signaling Technology, Inc., Beverly, MA) and anti-actin antibody (Sigma-Aldrich Co. Ltd.). The integrated signal density of each protein band was analyzed by the C-DiGit® Blot Scanner System (LI-COR Biosciences, Lincoln, NE). For evaluating phospho-ERK levels, ratios of the signal intensities of pERK/tERK were calculated.

Statistical analysis

All results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The data were subjected

to ANOVA or unpaired *t*-test to determine differences (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). If differences were detected by ANOVA, Fisher's protected least significant difference (PLSD) test was used to determine which means differed (StatView 5.0 software). *P* values < 0.05 were accepted as statistically significant.

Results

Expression of melatonin MT2 receptor mRNA in GH3 cells was clearly detected by RT-PCR (**Fig 1A**, *left panel*), whereas MT1 expression was very weakly detected. Both MT1 and MT2 receptor transcripts were expressed in the rat whole pituitary tissues (**Fig. 1A**, *right panel*). As shown in **Fig. 1B**, treatment with FSK (1 μM) for 24 h significantly increased mRNA expression of PRL, which was suppressed by co-treatments with melatonin and the MT1/M2 agonist ramelteon, in a concentration-responsive manner (10-100 μM). The inhibitory effect of ramelteon on PRL mRNA expression was more potent than that of melatonin (**Fig. 1B**). As shown in **Fig. 1C**, the reduction of PRL mRNA expression caused by melatonin (100 nM) was reversed in the presence of the MT1/MT2 antagonist luzindole (100-1000 nM), suggesting functional involvement of MT2 action in the suppression of PRL expression by GH3 cells. Furthermore, treatment with melatonin (100-1000 nM) (**Fig. 1D**) and treatment with ramelteon (**Fig. 1E**) suppressed FSK (1 μM)-induced PRL secretion in the culture medium for 24 h. Basal PRL secretion was significantly decreased by ramelteon (**Fig. 1E**) but not by melatonin (100-1000 nM) (**Fig. 1D**).

It has been reported that BMP-4 increases PRL synthesis via the crosstalk of Smad/ER complex [16] and through an increase of cellular cAMP synthesis by GH3 cells [18]. Here we examined the involvement of BMP-4 signaling in the actions of melatonin on PRL expression and secretion. As shown in **Fig. 2A**, BMP-4 stimulation (100 ng/ml) for 1 h readily activated Smad1/5/8 phosphorylation in GH3 cells, and pretreatment with melatonin (100 nM) or with

ramelteon (100 nM) suppressed BMP-4-induced Smad1/5/8 phosphorylation in 24-h culture. Under the condition of co-treatment with luzindole (100 nM), the inhibitory effects of melatonin and ramelteon on BMP-induced Smad activation were reversed (**Fig. 2A**), suggesting functional involvement of MT2 action in the suppression of Smad1/5/8 signaling in GH3 cells. To confirm the effects of melatonin receptor activation on BMP-receptor signaling, mRNA levels of the target gene of BMP-receptor signaling, Id-1, were evaluated. As shown in **Fig. 2B** (*left panel*), treatment with BMP-4 (100 ng/ml) significantly increased Id-1 mRNA expression for 24 h, which was further upregulated in the presence of FSK (1 μM). Of note, treatment with ramelteon (100 nM) suppressed Id-1 mRNA expression induced by BMP-4 (100 ng/ml) and FSK (1 μM). As shown in **Fig 2B** (*right panel*), PRL mRNA level was increased by treatment with BMP-4 (100 ng/ml), FSK (1 μM) or the combination of BMP-4 and FSK for 24 h. In accordance with the suppressive effects of ramelteon on Smad and Id-1 signaling, the induction of PRL mRNA expression by treatments with FSK (1 μM) and BMP-4 (100 ng/ml) was significantly suppressed by treatment with ramelteon (100 nM) for 24 h (**Fig 2B**, *right panel*). In order to know the role of MT2 function in GH3 cells, we also examined the expressional changes of MT2 caused by BMP-4, which is an inducer of PRL expression. As shown in **Fig. 2C**, BMP-4 treatment upregulated MT2 mRNA expression for 24 h, implying the presence of a counter-regulating effect of BMP-4 for controlling PRL level via MT2 action.

We next examined the intracellular signaling involved in the melatonin receptor activation in GH3 cells. As shown in **Fig. 3A**, melatonin and ramelteon treatments significantly reduced basal cAMP synthesis for 24-h culture.

Ramelteon, but not melatonin, also suppressed BMP-4-induced cAMP synthesis for 24 h. Ramelteon, but not melatonin, treatment reduced FSK-induced cAMP production for 24 h (**Fig. 3B**). Co-treatment with BMP-4 and FSK further increased cAMP synthesis, which was also suppressed by ramelteon (**Fig. 3B**), suggesting that MT2 signaling is functionally involved in the suppression of cAMP synthesis in GH3 cells. In contrast, as shown in **Fig. 3C**, melatonin or ramelteon treatment failed to suppress BtcAMP-induced PRL mRNA expression.

Furthermore, the involvement of MAPK pathways in PRL regulation by melatonin was examined. As can be seen in **Fig. 3D**, phosphorylation of the ERK pathway induced by 1-h stimulation with FSK (1 μM) was clearly shown by Western blots, compared with that of P38 and SAPK/JNK pathways in GH3 cells. Also, as shown in the relative intensity of pERK/tERK, the FSK-induced ERK phosphorylation was significantly impaired by 24-h pretreatment with ramelteon but not melatonin (100 nM) (**Fig. 3D***, lower panel*). In order to determine the functional involvement of these signaling pathways, specific inhibitors (1 μM) for cAMP-PKA and MAPK pathways were utilized for examining the changes of PRL mRNA levels. As shown in **Fig. 3E**, treatment with a cAMP-PKA inhibitor, H-89, and treatment with an ERK inhibitor, U0126, significantly suppressed FSK-induced PRL mRNA expression for 24 h. These results suggested that not only cAMP-PKA signaling but also ERK signaling is, at least in part, involved in the melatonin receptor activity for regulating PRL expression in GH3 cells.

Discussion

In the present study, a regulatory interaction of melatonin and BMP-4 in PRL production was uncovered in pituitary lactotrope GH3 cells (**Fig. 4**). Melatonin receptor activation, induced by melatonin and its receptor agonist ramelteon, suppressed basal and FSK-induced PRL production. Melatonin actions are elicited via G protein-coupled receptors, MT1 and MT2, which are expressed in the brain and various peripheral tissues [2]. It has been reported that MT1, but not MT2, was highly expressed in human [26] and non-human primate pituitaries [27], suggesting that MT actions at the pituitary level in primates might be exclusively mediated through MT1. In contrast, MT2 is the major subtype in GH3 cells compared with the whole rat pituitary, which expresses both MT1 and MT2. Melatonin activity may also be elicited via a receptor-unrelated mechanism; however, the inhibitory actions of melatonin on PRL synthesis were restored by co-treatment with luzindole, suggesting that suppressive effects of melatonin on PRL release are functionally mediated via MT2 expressed on GH3 cells. Moreover, the finding that a PRL inducer, BMP-4, increased MT2 expression also suggested the existence of a negative feedback loop for regulating PRL secretion via the upregulation of MT2.

As for the effects of melatonin on lactosomatotrope functions, inhibitory actions of melatonin on PRL secretion in fish pituitary tissues was previously reported [28]. It was shown by using radiolabeled iodomelatonin that a melatonin analog bound selectively to membrane preparations of trout pituitaries. Melatonin inhibited FSK-induced cAMP accumulation, and in the absence of FSK, melatonin decreased PRL release through a direct action on pituitary cells. Using primary cultured prolactinoma cells derived from an estrogen-induced rat pituitary

PRL-secreting tumor, melatonin action was found to inhibit cell growth and induce apoptosis of prolactinoma cells [29]. Melatonin increased the activity of caspase-3 and the expression of Bax and cytochrome c in prolactinoma cells, while it suppressed Bcl-2 expression and mitochondrial membrane potential, indicating that melatonin inhibits tumor growth by inducing apoptosis of PRL-secreting tumor cells directly through mitochondrial damage.

Divergent effects of melatonin on pituitary cells from the pars distalis were also shown in experiments using primate pituitary cells [27]. In that study, melatonin was found to increase PRL release, whereas it did not affect the release of ACTH, gonadotropins or thyrotropin. The effects of melatonin on PRL release are likely to be mediated via MT1 actions, leading to the signaling pathways of adenylyl cyclase (AC)-PKA/extracellular calcium channels and PKC/intracellular calcium channels [27]. In the present study on GH3 cells, melatonin receptor activation, mainly via MT2, suppressed basal, BMP-4-induced and FSK-induced PRL secretion. These effects were accompanied by reductions of basal, BMP-4-induced and FSK-induced cAMP levels and suppression of FSK-induced ERK phosphorylation; however, the PRL induction directly stimulated by BtcAMP, via PKA, was not affected by treatment with melatonin or ramelteon. These findings suggest that MT2 activation leads to inhibition of PRL production through the suppression of BMP-receptor signaling as well as AC-induced cAMP synthesis at the upstream of PKA in GH3 cells.

In our earlier study on corticotrope cells [7], melatonin and its receptor agonist ramelteon suppressed CRH-induced pro-opiomelanocortin transcription and ACTH production. In such regulatory actions, MT1 expression was

upregulated by BMP-4, while BMP-4-induced Smad activity was increased by MT1 activation. BMP-4 and melatonin individually suppress CRH-induced ACTH production, and these two actions are mutually enhanced [7, 30], leading to elaborate regulation of ACTH secretion by corticotrope cells. Compared to these findings, in lactotrope cells, MT2 action was found to suppress PRL production by a dual mechanism for inhibiting BMP-4-induced Smad signaling and cAMP synthesis, resulting in effective suppression of PRL synthesis by MT2 activation. These results further suggested that melatonin substantially contributes to the establishment of daily patterns of circulating PRL, although species-dependent differences seem to exist in such melatonin actions. Considering that the activity of ramelteon, having 6-fold and 3-fold higher affinity to MT1 and MT2, respectively, than that of melatonin [24], was efficacious compared with the effect of melatonin in our experiments, the suppressive effect on PRL production is likely to be mediated via MT2 signaling in GH3 cells.

There has been an accumulation of information on expressional and functional relationships between melatonin and TGF-β molecules. Melatonin increases TGF-β synthesis in human prostate epithelial cells, leading to melatonin-mediated attenuation of cell proliferation [31]. Inhibition of breast cancer cell proliferation by melatonin with vitamin D3 is linked to activation of Smads [32]. Melatonin stimulates an osteogenic process by promoting the expression of BMP-2 and -4 through ERK and Wnt pathways [33]. In ovarian granulosa cells, melatonin was shown to suppress BMP-6-induced Smad signaling by reducing Smad6 expression [34]. Melatonin also appeared to be

involved in the fine tuning of steroidogenesis and catecholaminogenesis by modulating the BMP/activin system in the adrenal gland [35, 36].

BMP-4 has been shown to be overexpressed in lactotrope adenomas derived from D2 receptor-null mice, in estrogen-treated female rat pituitary tissues, and in human prolactinomas with its expression levels in other types of pituitary tumors [15]. In addition, type I and type II receptors for BMPs each exhibit serine/threonine kinase activity, in which several preferential combinations of BMP ligands and receptors have been recognized. Since ALK-6 is not expressed in GH3 cells, the pair of ALK-3 and BMPRII is likely to be a major functional complex for BMP-4 for regulating PRL and cAMP production [17]. Biologically, BMP-4 promotes lactotrope cell proliferation [15] and PRL production in conjunction with Smad-ER interaction [16]. In the present study, MT2 activity was found to be a suppressor of PRL production partly through inhibition of BMP-4 activity in lactotrope cells. The finding that BMP-4 upregulated MT2 expression in GH3 cells may indicate the existence of a feedback action of BMP-4 for regulating PRL level through MT2 activation. A possible regulatory mechanism for the interaction of BMP-4-Smad and ER and its critical binding sites have been identified in the PRL promoter region [16]. In a future study, detailed analysis using PRL-promoter constructs and primary lactotrope cells might lead to clarification of the molecular interaction between melatonin and BMP-4 signalings.

Collectively, the results revealed that melatonin agonists have suppressive effects on PRL secretion and BMP-4 signaling. The results suggested that melatonin acts as a functional modulator for the pituitary BMP system that can

induce PRL secretion (**Fig. 4**). Prolactinomas are the most frequent human functioning pituitary tumors. Administration of dopamine agonists is the main choice for treatment of most prolactinomas; however, some cases of prolactinomas have been shown to be dopamine-resistant [37]. Upregulation of melatonin effects and downregulation of the endogenous BMP system, which are the functional determinants of PRL release, could be an alternative strategy for medication control of pituitary adenomas [14]. An attempt to upregulate MT2 bioavailability may be clinically useful for the control of pituitary prolactinomas.

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Figure Legends:

Fig. 1. Expression of melatonin receptors and effects of melatonin on PRL secretion by GH3 cells. A) The expression of mRNAs encoding MT1, MT2 and the housekeeping gene RPL19 was examined by RT-PCR analysis in GH3 cells compared with that in a control sample extracted from the rat pituitary. MM indicates molecular weight marker. **B, C**) GH3 cells (3×10^5 cells/well) were precultured in serum-free DMEM/F12. The cells were treated with the indicated concentrations of forskolin (FSK), melatonin (Mel), ramelteon (Ram) and luzindole (Luz) in serum-free media. After 24-h culture, total cellular RNA was extracted and subjected to RT-PCR reaction. The mRNA expression levels of PRL were quantified by real-time PCR analysis. The expression levels of target genes were standardized by RPL19 level in each sample. **D**, **E**) Cells (1×10^5 cells/well) were precultured in serum-free DMEM/F12. The cells were then treated with FSK in combination with **D**) Mel or **E**) Ram. After 24-h culture, the supernatants of culture media were collected, and PRL levels were determined by specific enzyme immunoassays. 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 or the unpaired *t*-test. For each result within a panel, *, $P < 0.05$ and **, $P < 0.01$ vs. control group or between the indicated groups; #, $P < 0.05$ vs. the FSK-treated group; and the values with different superscript letters are significantly different at $P < 0.05$.

Fig. 2. Effects of melatonin receptor activation on BMP-4 signaling in GH3 cells. **A**) GH3 cells (3×10^5 cells/well) were pre-treated with the indicated concentrations of FSK, Mel, Ram and Luz in serum-free media for 24 h. After 1-h stimulation with BMP-4, the cells were lysed and subjected to immunoblot (IB) analysis using antibodies that detect pSmad1/5/8 and actin. The results are representative of results obtained from three independent experiments. **B, C**) Cells (3×10^5 cells/well) were treated with the indicated concentrations of FSK, BMP-4, Mel and Ram in serum-free conditions for 24 h. Total cellular RNAs were extracted and the mRNA levels of Id-1, PRL and MT2 were examined by real-time PCR. The expression levels of target genes were standardized by RPL19 level in each sample. 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 (**B**) or the unpaired *t*-test (**C**). For each result within a panel, the values with different superscript letters are significantly different at $P < 0.05$. *, $P < 0.05$ between the indicated groups.

Fig. 3. Involvement of cAMP-PKA and MAPK in the suppression of PRL production by melatonin receptor activation. **A, B**) GH3 cells (1×10^5 cells/well) were precultured in serum-free DMEM/F12. The cells were then treated with Mel, Ram, and BMP-4 in the **A**) absence or **B**) presence of FSK with serum-free medium containing 0.1 mM of IBMX. After 24-h culture, the supernatants of culture media were collected, and cAMP levels were determined by specific enzyme immunoassays. **C**, **E**) Cells (3×10^5 cells/well) were treated with the indicated concentrations of BtcAMP, FSK, Mel, Ram, and each inhibitor

of intracellular cAMP-PKA and MAPKs including H-89, U0126, SP203580 and SP600125 in serum-free DMEM for 24 h. Total cellular RNAs were extracted and the mRNA levels of PRL were examined by real-time PCR. The expression levels of target genes were standardized by RPL19 level in each sample. Results in all panels are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. **D**) Cells (3×10^5 cells/well) were pretreated with the indicated concentrations of Mel or Ram in serum-free conditions for 24 h. After 1-h stimulation with an indicated concentration of FSK, the cells were lysed and subjected to immunoblot (IB) analysis using antibodies that detect phosphorylation of MAPKs. The relative integrated density of the ERK protein band was digitized, with the phosphorylated levels being normalized by the total levels (pERK/tERK), and then the results were 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 (**A**-**D**) and the unpaired *t*-test (**E**). For each result within a panel, the values with different superscript letters are significantly different at $P < 0.05$; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control; and n.s.: not significant.

Fig. 4. Functional interaction of melatonin and BMP-4 for regulating PRL secretion in lactotrope cells. Melatonin MT2 receptor expression was predominant compared with MT1 receptor expression in lactotrope GH3 cells. MT2 receptor activation suppressed basal and FSK-induced PRL mRNA expression and PRL secretion with reductions in cAMP synthesis via adenylyl cyclase (AC) and MAPK (ERK) activity. Melatonin action also inhibited BMP-4-

induced PRL expression by inhibiting BMP-induced Smad1/5/8 phosphorylation and transcription of the BMP-target gene Id-1. On the other hand, BMP-4 increased MT2 expression on GH3 cells. These results suggested that melatonin MT2 activity plays a regulatory role in PRL secretion through suppression of cAMP-PKA and BMP-4 signaling in lactotrope cells.

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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; BtcAMP, N^6,O^2-dibutyryl adenosine-3',5'-cyclic monophosphate monosodium salt; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; FSK, forskolin; JNK, c-Jun NH2-terminal kinase; Luz, luzindole; MAPK, mitogen-activated protein kinase; Mel, melatonin; PRL, prolactin; Ram, ramelteon; SAPK, stress-activated protein kinase; and TGF-β, transforming growth factor-β.