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Interaction of pituitary hormones and expression of clock genes modulated by bone morphogenetic protein-4 and melatonin

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ABSTRACT

Functional interaction of clock genes and pituitary hormones was investigated by focusing on bone morphogenetic protein (BMP)-4 and melatonin actions in anterior pituitary cells. A significant correlation between the mRNA expression of proopiomelanocortin (POMC) and Per2 was revealed in serial cultures of corticotrope AtT20 cells. Knockdown of Per2 expression by siRNA in AtT20 cells resulted in a significant reduction of POMC mRNA level with or without corticotropin-releasing hormone (CRH) stimulation. Treatments with BMP-4 and melatonin, both of which suppress POMC expression, reduced Per2 mRNA as well as protein levels in AtT20 cells. On the other hand, in lactosomatotrope GH3 cells, an expressional correlation was found between prolactin (PRL) and Clock mRNA levels, which was attenuated in the presence of forskolin treatment. The siRNA-mediated knockdown of Clock expression, but not that of Bmal1, significantly reduced PRL mRNA levels in GH3 cells. Interestingly, Clock mRNA and protein levels did not fluctuate with melatonin, BMP-4 or forskolin treatment, although Bmal1 expression was significantly increased by forskolin treatment. Collectively, a significant correlation between the expression of POMC and Per2 and that between PRL and Clock were uncovered in corticotrope and lactosomatotrope cells, respectively. Per2 expression was inhibited by POMC modulators including melatonin and BMP-4, while Clock expression was steadily maintained. Thus, the effects of melatonin and BMP-4 on clock gene expression may imply differential stability of circadian rhythms of adrenocorticotropin (ACTH) and PRL secreted from the anterior pituitary.

1. Introduction

The circadian pacemaker is known to regulate physiological rhythm through neurochemical and hormonal transmitters by coordinating the oscillations of peripheral clocks that reside in various organs [1]. As has been shown in peripheral tissues, the pituitary has also been thought to involve the molecular clock that is capable of capturing and sensitizing time autonomously [2,3]. A recent study by Wunderer and colleagues [4] has shown the existence of pituitary clock gene activity in human pituitaries, although it remains inconclusive whether the clock gene activity is functional or not. In this regard, Becquet et al. [5] demonstrated that core-clock genes exhibit a certain expression pattern using rat primary pituitary cells synchronized by forskolin (FSK), indicating the presence of a functional circadian oscillator in the pituitary. These findings suggest that pituitary cells contain the endogenous circadian system. However, the physiological role and the regulatory mechanism of this intrinsic clock in the pituitary have yet to be clarified.

There are various functional regulators such as growth factors and cytokines expressed in the pituitary. Among these, bone morphogenetic proteins (BMPs), which exert various activities on endocrine tissues [6], have been shown to play important roles in the initial development of the anterior pituitary [7]. BMP-4 not only governs pituitary organogenesis but also contributes to the

Abbreviations: ACTH, adrenocorticotropin; BMP, bone morphogenetic protein; CRH, corticotropin-releasing hormone; Cry, cryptochrome; FSK, forskolin; LH, luteinizing hormone; Per, period; POMC, proopiomelanocortin; PRL, prolactin; SCN, suprachiasmatic nucleus.

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pathogenesis of differentiated pituitary lineages [8–10]. BMP-4 was found to be overexpressed in lactotrope adenomas derived from rodent models and also in human prolactinomas [11]. It is of note that bioactivity of BMP action was shown in corticotrope cells as a negative regulator of proopiomelanocortin (POMC) expression, adrenocorticotropin (ACTH) secretion and cell proliferation [12–15]. Interestingly, the expression levels of BMP-4 are reduced in human corticotrope adenomas of Cushing's disease compared with the levels in normal pituitary tissues [13].

Melatonin is a lipophilic indoleamine synthesized from serotonin by the pineal gland. Melatonin acts as a hormonal regulator for circadian rhythm in conjunction with the suprachiasmatic nucleus (SCN) and peripheral tissues, in which circulating levels of melatonin are low in the daytime and high at night [3]. However, in Cushing's syndrome, exhibiting a lack of circadian rhythm, the circadian change of melatonin was shown to be out of tune [16]. We previously reported that melatonin action via MT1 receptors augments BMP-4 receptor signaling in corticotrope cells, resulting in effective suppression of POMC transcription and ACTH production [17].

In the present study, we focused on the effects of BMP-4 and melatonin on the expression patterns of key clock genes, including Bmal, Clock, Period (Per) and Cryptochrome (Cry), that are possibly involved in controlling circadian rhythm in the regulatory process of ACTH and prolactin (PRL) production. The differential effects of BMP-4 and melatonin on clock gene expression may reflect the stability or accuracy of circadian fluctuations of ACTH and PRL secretion from the pituitary.

2. Materials and methods

2.1. Reagents and supplies

Human and rat CRH, melatonin and forskolin (FSK) were purchased from Sigma–Aldrich Corp. (St. Louis, MO). Recombinant human BMP-4 was purchased from R&D Systems Inc. (Minneapolis, MN).

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

Mouse pituitary corticotrope AtT20/D16v (AtT20) cells (1×10^5 viable cells) were treated with indicated concentrations of BMP-4 and melatonin alone or in combination with CRH in serum-free DMEM. Rat pituitary lactosomatotrope tumor GH3 cells (1×10^5 viable cells) were treated with indicated concentrations of BMP-4 and melatonin alone or in combination with FSK in serum-free DMEM/F12. After the indicated culture periods, total cellular RNAs were extracted using TRIzol® (Invitrogen Corp., Carlsbad, CA). The extracted RNA (1 μg) was subjected to RT reaction using the 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 70 °C for 10 min. Primer pairs for mouse POMC, PRL and RPL19 were selected as reported previously [14,15,17,18]. The other primer pairs were selected from different exons of the corresponding genes as follows: 428-448 and 621-642 for Bmal1 (from GenBank accession #AB015203); 1875-1896 and 1975-1996 for Clock (NM_007715); 683-705 and 871-893 for Per2 (NM_011066); and 1638-1660 and 1739-1763 for Cry1 (NM_007771). For the quantification of mRNA levels of target genes, real-time PCR was performed using the LightCycler® Nano Real-Time PCR system (Roche Diagnostic Co., Tokyo, Japan) under optimized annealing conditions at 60–62 °C. The relative expression of each mRNA was calculated by the Δ threshold cycle (Ct) method, in which ΔCt was 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 results were expressed as the ratio of target mRNA to RPL19 mRNA.

2.3. Transient transfection of siRNA

AtT20 and GH3 cells (1×10^5 viable cells) were cultured in 12-well plates in 1 ml of individual growth medium supplemented with 10% FCS without antibiotics. Cells were transiently transfected with 10 μM target gene siRNA (30 pmol/well) including Per2, Bmal1 and Clock, or control siRNA, duplex using the transfection medium and reagents following the protocol of the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA). After 7-h transfection, cells were treated by adding 0.5 ml of normal growth medium containing two times the regular FCS and antibiotics and the cells were incubated for 24 h. After culture for indicated periods, total cellular RNA was isolated. The extracted RNA was subjected to RT reaction, and real-time PCR was performed for the quantification of mRNA levels of siRNA-target genes, POMC and PRL as described.

2.4. Western immunoblot analysis

AtT20 cells and GH3 cells (1×10^5 viable cells/well) were treated with CRH, FSK, BMP-4 and melatonin either alone or in the indicated combinations in serum-free medium. After stimulation for 6 or 24 h, 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. Cells were 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-Bmal1, Clock and Per2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) 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, NE). For evaluating the target protein levels, ratios of the signal intensities of the target protein/actin were calculated.

2.5. Statistical analysis

Results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. Differences between groups were analyzed for statistical significance using ANOVA with Tukey–Kramer's post hoc test or the unpaired *t*-test, when appropriate, to determine differences, and correlation of each variable was assessed by using simple regression analysis (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). *P* values <0.05 were accepted as statistically significant.

3. Results

First, we investigated the existence of an expressional correlation of POMC and clock genes in mouse corticotrope AtT20 cells. The mRNA levels of POMC and clock genes, including Bmal1, Clock, Per2 and Cry1, were examined in AtT20 cells serially cultured for 6–48 h. As shown in Suppl. Fig. 1, linear regression analysis uncovered a significant correlation between the mRNA expression levels of POMC and Per2 ($R^2 = 0.5$, $^{**}P < 0.01$) in the 24-h culture condition. As shown in Fig. 1A, the correlation between POMC and Per2 mRNA levels for 24 h was apparent ($R^2 = 0.4$, $^{**}P < 0.01$) in the condition of culture without CRH (100 nM) treatment.

To determine the functional role of Per2 expression in corticotrope cells, POMC mRNA levels were evaluated in AtT20 cells in which Per2 expression was repressed by siRNA targeting. As shown

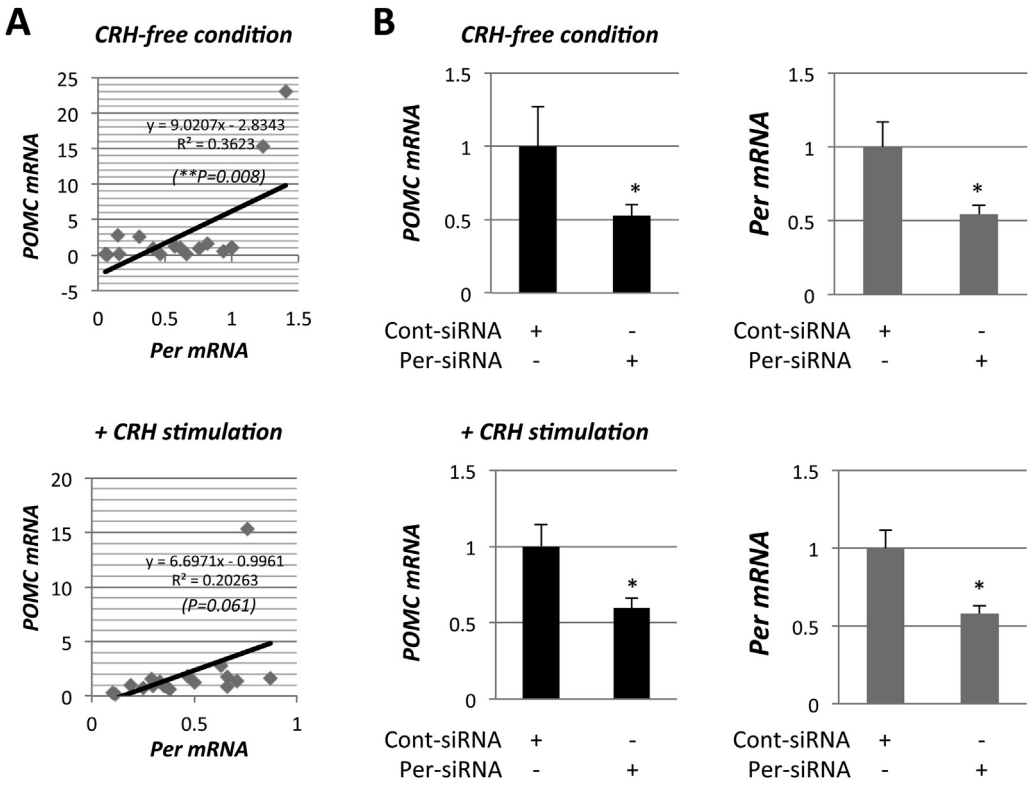

Fig. 1. Effects of Per inhibition on POMC expression in corticotrope cells. A) AtT20 cells were cultured in a serum-free condition in the presence or absence of CRH. After 24-h culture, total cellular RNAs were extracted and the expression levels of target gene mRNA were standardized in each sample, and then linear regression analysis was performed between the mRNA expression levels of POMC and Per2. $^{**}P < 0.01$ of the significant correlation (n = 18). **B**) AtT20 cells were transiently transfected with 10 μM target-gene siRNA for Per2 or control siRNA and incubated for 24 h in the presence or absence of CRH. Total cellular RNAs were then extracted and the mRNA levels of POMC and Per2 were examined. Results are shown as means ± SEM. The results were analyzed by the unpaired *t*-test. $^{*}P < 0.05$ vs. control groups.

in Fig. 1B, the knockdown of Per2 expression by siRNA significantly reduced Per2 mRNA levels by 46% and 42% in CRH-free and CRH (100 nM)-stimulated conditions, respectively, for 24-h treatments. The Per2 suppression resulted in significant and potent reductions of POMC mRNA in AtT20 cells by 47% and 40% in the corresponding conditions, respectively.

We also examined the effects of BMP-4 (100 ng/ml) and melatonin (30 nM), both of which suppress POMC expression and ACTH production in AtT20 cells, on POMC mRNA expression by AtT20 cells. As shown in Fig. 2A, BMP-4 (100 ng/ml) reduced basal as well as CRH (100 nM)-induced POMC expression, while melatonin (30 nM) significantly suppressed CRH-induced POMC expression for 24 h. Of note, treatment with either BMP-4 (100 ng/ml) or melatonin (30 nM) enabled reduction of Per2 mRNA levels for 24 h. BMP-4 (100 ng/ml) and melatonin (30 nM) also suppressed Per2 mRNA expression in the presence of CRH (100 nM), while CRH alone had an inhibitory effect on Per2 expression in AtT20 cells. In accordance with the changes in Per2 mRNA levels, CRH (100 nM), BMP-4 (100 ng/ml) and melatonin (30 nM) individually suppressed Per2 protein expression in AtT20 cells for 24 h (Fig. 2B).

Next, the expressional correlations of PRL and clock genes were examined using rat lactosomatotrope GH3 cells cultured for 6–48 h. As shown in Suppl. Fig. 2, correlations were found between mRNA expression levels of PRL and Clock ($R^2 = 0.7$, $^{**}P < 0.01$) and those of PRL and Bmal1 ($R^2 = 0.3$, $^{**}P < 0.05$) in a 6-h culture condition. As shown in Fig. 3A, the correlation between PRL and Bmal1 expression was attenuated in the presence of FSK (1 μM). The siRNA-targeting knockdown of Bmal1 gene expression by 50–60% reduction did not affect PRL mRNA levels regardless of the presence or absence of FSK. As shown in Fig. 3B, the correlation between PRL and Clock expression was also prominent ($R^2 = 0.7$, $^{**}P < 0.01$) in the condition of culture without FSK treatment. The knockdown of Clock gene expression by siRNA transfection, leading to 54–58% reduction of Clock mRNA level, significantly decreased PRL mRNA levels without, but not with, FSK (1 μM) treatment in GH3 cells for 24 h.

As shown in Fig. 4A, BMP-4 (100 ng/ml) stimulation moderately upregulated PRL and Bmal1 mRNA levels for 6 h, while it did not affect Clock mRNA levels. FSK (1 μM) induced PRL and Bmal1, but not Clock, mRNA expression. Melatonin (30 nM) increased Bmal1 mRNA expression but reduced FSK-induced PRL mRNA expression for 6 h. In addition, melatonin alone had no effect on Clock mRNA level, but it moderately increased Clock expression in the presence of FSK for 6 h.

The expressional changes of Bmal1 and Clock proteins were further confirmed by Western blots (Fig. 4B). FSK (1 μM) treatment significantly increased Bmal1 protein expression for 6 h, and treatment with BMP-4 (100 ng/ml) or melatonin (30 nM) showed moderate effects on Bmal1 protein induction. In contrast, Clock protein levels were not changed by treatment with FSK, BMP-4 or melatonin.

4. Discussion

The anterior pituitary is composed of five different cell lineages including corticotrope and lactotorope cells that show endocrine activities with their proper patterns and rhythms. The cell lineages in the anterior pituitary are mainly under control of the corresponding hypothalamic hormones. However, it has been thought that the pituitary cells also contain their own molecular clock that

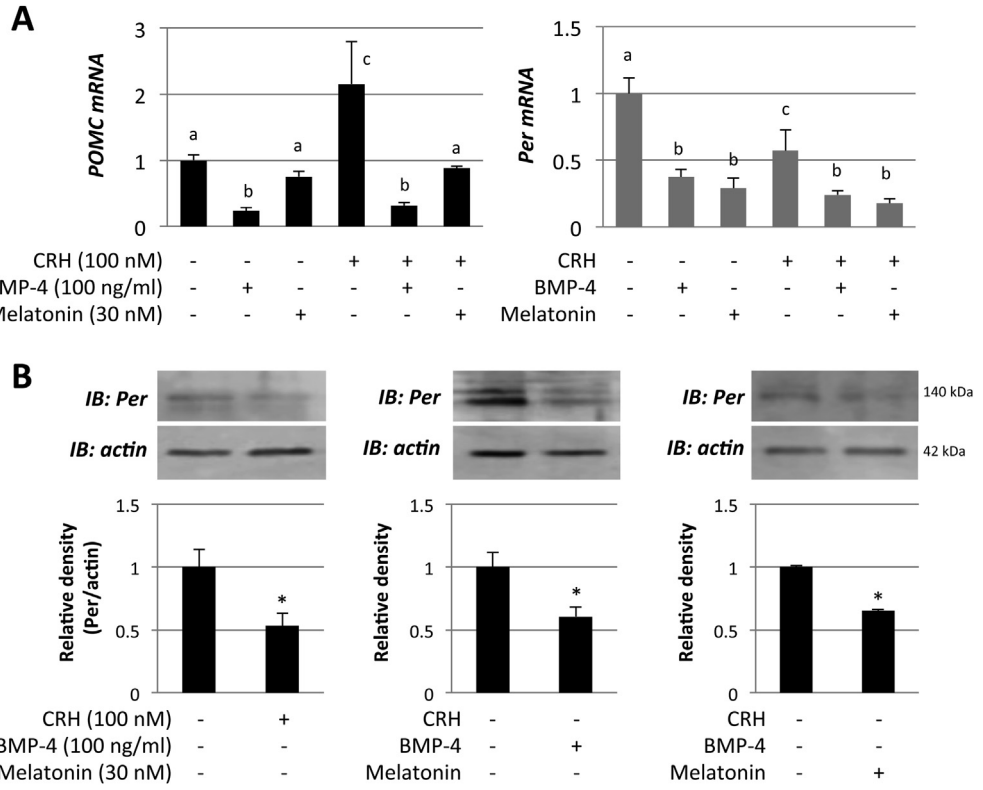

Fig. 2. Effects of CRH, BMP-4 and melatonin on POMC and Per expression in corticotrope cells. A) AtT20 cells were treated with CRH, BMP-4 and melatonin in a serum-free condition. After 24-h culture, total cellular RNAs were extracted and the expression levels of target gene mRNA were standardized in each sample. Results are shown as means ± SEM. The values with different superscript letters are significantly different at $P < 0.05$. **B**) After 24-h culture, cell lysates were extracted and were subjected to immunoblotting for Per2 and actin analysis. The relative integrated density of each protein band was digitized, and Per2 levels were normalized by actin in each sample. Results are shown as means ± SEM. The results were analyzed by the unpaired *t*-test. $^{*}P < 0.05$ vs. control groups.

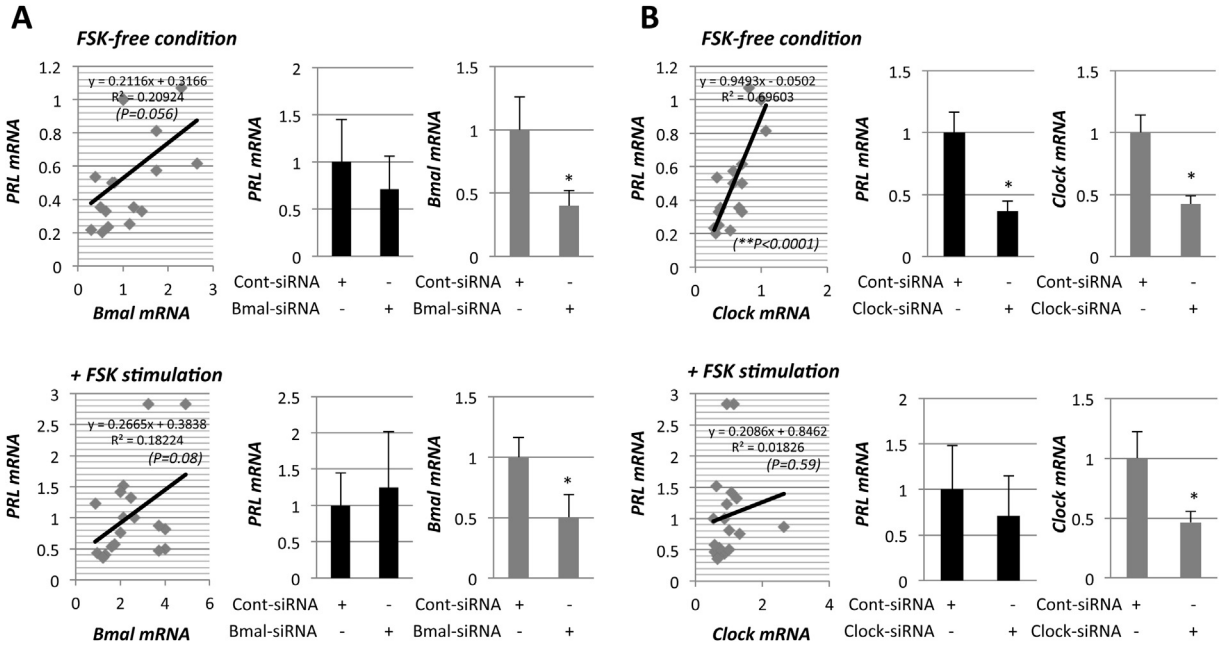

Fig. 3. Effects of Bmal and Clock inhibition on PRL expression in GH3 cells. GH3 cells were cultured in a serum-free condition in the presence or absence of FSK. After 6-h culture, total cellular RNAs were extracted and the expression levels of target gene mRNA were standardized in each sample, and then linear regression analysis was performed for correlations between the mRNA levels of **A**) Bmal1 and PRL or **B**) Clock and PRL. $^{**}P < 0.01$ of the significant relationship (n = 24). Cells were also transiently transfected with 10 μM target gene siRNA for **A**) Bmal1, **B**) Clock, or control siRNA and incubated for 24 h in the presence or absence of FSK. Total cellular RNAs were then extracted and the mRNA levels of PRL, Bmal1 and Clock were examined. Results are shown as means ± SEM. $^{*}P < 0.05$ vs. control groups.

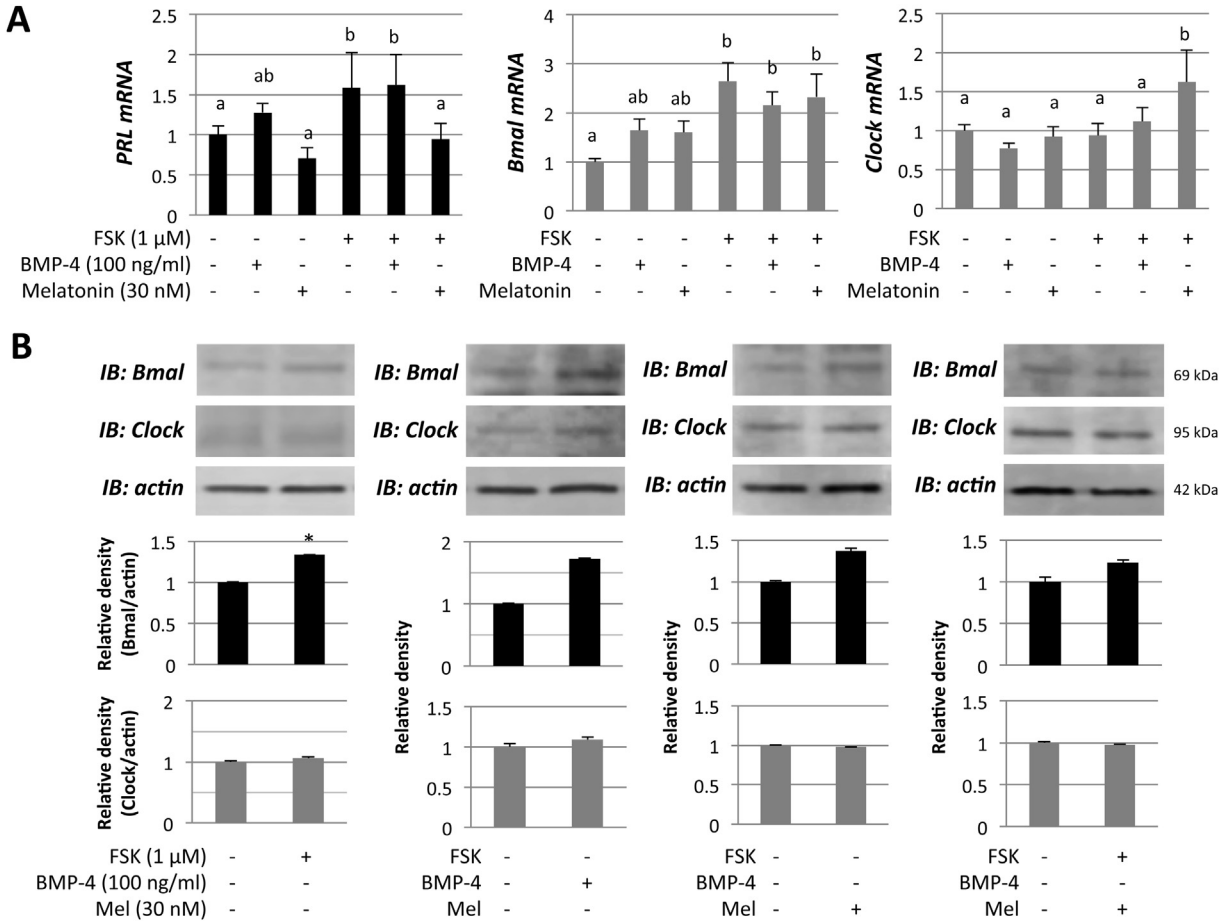

Fig. 4. Effects of FSK, BMP-4 and melatonin on PRL, Bmal and Clock expression in lactosomatotrope cells. A) GH3 cells were treated with FSK, BMP-4 and melatonin in a serum-free condition. After 6-h culture, total cellular RNAs were extracted and the expression levels of target gene mRNA were standardized in each sample. Results are shown as means ± SEM. The values with different superscript letters are significantly different at $P < 0.05$. **B**) After 6-h culture, cell lysates were extracted and were subjected to immunoblotting for Bmal1, Clock and actin analysis. The relative integrated density of each protein band was digitized, and Bmal1 and Clock levels were normalized by actin in each sample. Results are shown as means ± SEM. $^{*}P < 0.05$ vs. control groups.

is autonomously capable of oscillating. In the present study, the expressional correlation between POMC and Per2 gene at 24 h and that between PRL and Clock gene at 6 h were uncovered in pituitary corticotrope and lactosomatotrope cells, respectively (Suppl. Fig. 3). Moreover, it was found that the regulatory modes of clock gene expression were different in corticotrope and lactosomatotrope cells.

To maintain the homeostasis in response to changing internal and external circumstances, the pituitary needs to rely on time information. Abe and colleagues [2] previously provided evidence of the existence of circadian oscillators in the pituitary extracted from rats carrying the Per-luciferase transgene. Wunderer et al. [4] further explored the existence of clock genes and the day–night patterns in human pituitary tissues. Among the clock genes examined in human autopsied pituitaries, Per1 mRNA expression showed daytime-dependent differences according to time of death, while the protein levels of Per1, Cry1, and Clock did not fluctuate with time of day [4]. Becquet et al. [5] utilized rat pituitary primary culture and lactosomatotrope GH4C1 cells, showing the existence of a functional circadian oscillator in the pituitary. Disruption of the cellular oscillation by a dominant-negative Bmal1-R91A mutant resulted in loss of the circadian pattern of clock-controlled genes, suggesting the presence of a local oscillator in the pituitary for generating independent circadian rhythmicity. In view of reproductive functions in the pituitary, a lack of proestrous luteinizing hormone (LH) surge has been reported in Clock-mutant mice [19] and Bmal1-deficient female mice [20], suggesting that Clock and Bmal1 play a key regulatory role in gonadotoropin release from the pituitary.

In the present study, we demonstrated differential plasticity of clock gene expression in corticotrope and lactosomatotrope cells. That is, Per2 expression was inhibited by CRH, BMP-4 and melatonin in corticotrope cells, while Clock expression, in comparison to Bmal1, was not significantly altered by FSK, BMP-4 or melatonin in lactosomatotrope cells. The circadian expression of POMC in corticotrope cells is likely to be influenced by various environmental factors compared with that of PRL in lactotrope cells. The differential effects of BMP-4 and melatonin on clock gene expression may be physiologically associated with plasticity or stability of individual circadian fluctuations of ACTH and PRL secreted from anterior pituitary cells.

Correlation of the circadian system with PRL expression has been shown in the living rat lactotope cells [21] and in GH3 cells exposed to high concentrations of serum synchronizing the PRL-expression oscillation [22]. It was further revealed that PRL pulse activity is promoter-driven depending on a specific promoter-binding region of E-box [23,24]. In particular, the E-box133 site can bind a specific array of circadian components such as Bmal1,

Cry1 and Per1/3, in which circadian inputs are critical for PRL pulse activity [25]. In the present study, expressional correlations with PRL were revealed for mRNA level of Clock and weakly for mRNA level of Bmal1 at 6-h time points, wherein siRNA-mediated knockdown of Clock, but not that of Bmal1, significantly reduced PRL mRNA expression by GH3 cells. Considering that Clock expression was steadily preserved by treatment with BMP-4, melatonin or FSK, the circadian rhythm of PRL may be functionally preserved via Clock-mediated E-box activation.

Attention has recently been paid to the pars tuberalis within the pituitary gland as a key region of melatonin action [26], since MT1-expressing cells are abundantly located there. It has been proposed that melatonin drives a daily rhythm of gene expression coincident with dawn or dusk [27]. In this regard, West et al. [28] highlighted the function of Npas4, which plays an important role in melatonin-regulated timing circuits within the pituitary gland. In addition, melatonin inhibition of the proteasome appears to stabilize Bmal1 protein expression in the SCN, particularly at night, when melatonin level is normally elevated [29]. Melatonin inhibition of the proteasome may also account for the regulatory effects on circadian rhythm in the pituitary.

Based on the results of a previous study using a transgenic rat model expressing Per1-luciferase [2], the pineal and pituitary glands enable oscillations, being longer with a larger amplitude than other areas of the brain except for the SCN. Regarding the effects of pineal-derived melatonin in corticotrope cells, we previously reported that melatonin suppresses CRH-induced POMC transcription and ACTH production [17]. Considering that both BMP-4 and melatonin play inhibitory roles in CRH-induced POMC induction and that BMP-4 signaling and MT1 signaling are mutually enhanced in this mechanism [17], Per2 expression could be cooperatively repressed by BMP-4 and melatonin in corticotrope cells. Further study is necessary for elucidation of the pineal–pituitary interaction in the physiologic regulatory process of ACTH and PRL secretion. Although the physiological role of the pituitary clock within the circadian system is still inconclusive, the central clock in the SCN and humoral factors such as BMP-4 and melatonin may interact with functional oscillators present in the pituitary.

Collectively, the functional link between POMC, PRL and clock gene expression in pituitary cells was uncovered (Suppl. Fig. 3). Circadian regulation in pituitary hormones may, at least in part, occur at the level of the pituitary itself, under the influence of the autocrine/paracrine factor BMP-4 and pineal-derived melatonin, for fine-tuning and stabilization of pituitary hormone secretion in a time-coded manner.

Conflict of interest

The authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.100.

Transparency document

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