Mutual effects of melatonin and activin on induction of aldosterone production by human adrenocortical cells.

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Abbreviations:

ACTH, adrenocorticotropin
ACTH-R, adrenocorticotropin receptor
ActRI, activin type-I receptor
ActRII, activin type-II receptor
ALK, activin receptor-like kinase
Ang II, angiotensin II
AT1R, Ang II type 1 receptor
BMP, bone morphogenetic protein
BMPRI, BMP type-I receptor
BMPRII, BMP type-II receptor
CYP11B2, P450 aldo gene
CYP17, P450 c17 gene
IBMX, 3-isobutyl-1-methylxanthine
MAPK, mitogen-activated protein kinase
MR, mineralocorticoid receptor
MT, melatonin receptor
PKA, protein kinase A
TGF, transforming growth factor

Abstract

Melatonin has been reported to suppress adrenocorticotropin (ACTH) secretion in the anterior pituitary and cortisol production in the adrenal by different mechanisms. However, the effect of melatonin on aldosterone production has remained unknown. In this study, we investigated the role of melatonin in the regulation of aldosterone production using human adrenocortical H295R cells by focusing on the activin system expressed in the adrenal. Melatonin receptor MT1 mRNA and protein were expressed in H295R cells and the expression levels of MT1 were increased by activin treatment. Activin increased ACTH-induced, but not angiotensin II (Ang II)-induced, aldosterone production. Melatonin alone did not affect basal synthesis of either aldosterone or cortisol. However, melatonin effectively enhanced aldosterone production induced by co-treatment with ACTH and activin, although melatonin had no effect on aldosterone production induced by Ang II in combination with activin. These changes in steroidogenesis became apparent when the steroid

production was evaluated by the ratio of aldosterone/cortisol. Melatonin also enhanced dibutyryl-AMP-induced aldosterone/cortisol levels in the presence of activin, suggesting a functional link to the cAMP-PKA pathway for induction of aldosterone production by melatonin and activin. In accordance with the data for steroids, ACTH-induced, but not Ang II-induced, cAMP synthesis was also amplified by co-treatment with melatonin and activin. Furthermore, the ratio of ACTH-induced mRNA level of CYP11B2 compared with that of CYP17 was amplified in the condition of treatment with both melatonin and activin. In addition, melatonin increased expression of the activin type-I receptor ALK-4 but suppressed expression of inhibitory Smads6/7, leading to the enhancement of Smad2 phosphorylation. Collectively, the results showed that melatonin facilitated aldosterone production induced by ACTH and activin via the cAMP-PKA pathway. The results also suggested that mutual enhancement of melatonin and activin receptor signaling is involved in the induction of aldosterone output by adrenocortical cells.

Introduction

Aldosterone production in the adrenal glomerulosa is directly stimulated by angiotensin II (Ang II), potassium and adrenocorticotropin (ACTH). The major signal transduction pathway for ACTH stimulation of aldosterone production occurs through cAMP-protein kinase A (PKA), while Ang II action is transduced by diacylglycerol-protein kinase C, inositol 1,4,5-trisphosphate/Ca^{2+} signaling and mitogen-activated protein kinase (MAPK) via the Ang II type-I receptor (AT1R) [1].

In the presence of these major stimulators, adrenocortical steroidogenesis is modulated by local autocrine/paracrine factors that reside in adrenal tissues [2]. Basic fibroblast growth factor, insulin-like growth factors, and transforming growth factor (TGF)-β1 have been postulated to play roles in the regulation of adrenal steroidogenesis [2-5]. We previously reported the existence of a bone morphogenetic protein (BMP) and activin system consisting of specific type-I and -II receptors and Smads in adrenocortical cells [6-9].

TGF-β superfamily members including BMPs, growth and differentiation factors, and activins play important roles as autocrine/paracrine factors in the regulation of ovarian steroidogenesis [10, 11]. In adrenocortical cells, BMP-6 is involved in the stimulation of Ang II-induced aldosterone production by upregulating the MAPK pathway [7-9], while the activin system is functionally linked to the ACTH-induced cAMP-PKA cascade in adrenocortical cells [6]. In addition, BMP signaling in the adrenal medulla was also found to play a regulatory role in catecholamine synthesis induced by adrenocortical steroids [12, 13].

On the other hand, melatonin is involved in the physiological control of circadian and seasonal rhythms as well as in the activities of hormones and cytokines [14-16]. Melatonin actions are elicited via two types of G protein-coupled receptors, MT1 and MT2, which are expressed in the brain and various peripheral tissues. Melatonin receptors have also been detected in adrenal tissues. Regarding the effects of melatonin on adrenocortical hormones, it has been shown that melatonin, acting directly on the adrenal gland, inhibits the glucocorticoid response to ACTH in monkeys, sheep, rats and

humans [17-20].

The circadian rhythms of melatonin and ACTH are inversely fluctuated. In humans, melatonin secretion peaks at night and decreases in the daytime. In contrast, circulating ACTH-cortisol peaks in the early morning and declines during the night. Interestingly, in Cushing's syndrome, which exhibits a lack of ACTH-cortisol secretory rhythm, the circadian change of melatonin was shown to be abnormal [21]. This finding implies that the increased melatonin at night plays a physiological role in suppression of ACTH-cortisol secretion or that excessive cortisol may lead to the abolishment of normal melatonin rhythm. It has been revealed that melatonin inhibits ACTH-induced cortisol production via MT1 expressed on the adrenals in various mammals [17, 19, 20]. Given that melatonin secretion can be abnormally lower at night and higher in the daytime in patients with Cushing's syndrome [21], we assumed that the key circadian factor melatonin is involved in the pathogenesis of disturbed circadian changes in ACTH and cortisol.

Melatonin has been reported to suppress ACTH secretion in the

anterior pituitary and cortisol production in the adrenal by different mechanisms. However, the effect of melatonin on aldosterone production, in comparison with cortisol changes, has remained unknown. In order to clarify the interaction between melatonin and adrenal steroidogenesis under the influence of ACTH, we studied undefined roles of melatonin in the regulation of aldosterone production using human adrenocortical cells by focusing on the activin system.

Materials and Methods

Reagents and supplies

A 1:1 mixture of Dulbecco's Modified Eagle's Medium/Ham's F-12 medium (DMEM/F12), penicillin-streptomycin solution, and Ang II acetate salt adrenocorticotropic hormone human fragment 1-24 (1-24 ACTH), recombinant human activin A, N^6,O^2-dibutyryl adenosine-3',5'-cyclic monophosphate monosodium salt (BtcAMP), 3-isobutyl-1-methylxanthine (IBMX), melatonin and bovine serum albumin were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Insulin-transferrin-sodium selenite plus (ITS+) was from BD Falcon (Bedford, MA). Total human adrenal RNAs (Stratagene, San Diego, CA) were used as a control study.

Cell culture and hormone assays

The NCI-H295R human adrenocortical cell line was obtained from American Type Culture Collection (Manassas, VA). H295R cells were

maintained in DMEM/F12 supplemented with 10% FCS. Cells (3 x 10^5 viable cells/well) were precultured in 24-well plates with 10% FCS for 24 h. The medium was then changed to DMEM/F12 containing 1% FCS and 4 mM potassium, and the cells were treated with indicated reagents. After 24-h culture, aldosterone and cortisol concentrations in the culture media were measured by a radioimmunoassay (SPAC-S aldosterone, TFB Co., Tokyo) and chemiluminescent immunoassay (ACS-E Cortisol II, Siemens Healthcare Diagnostics Co., Tokyo), respectively. Steroid contents were undetectable in the cell-free medium. To assess cellular cAMP synthesis, cells (3 x 10^5 viable cells/well) were cultured in DMEM/F12 containing 1% FCS and 0.1 mM of a phosphodiesterase inhibitor, IBMX. After 24-h culture, the conditioned medium was collected and the extracellular contents of cAMP were determined by EIA (Cyclic AMP EIA Kit, Cayman Co., Ann Arbor, MI) with assay sensitivity of 0.3 nM.

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

Cells (5×10^5 viable cells) were grown in 12-well plates and the medium was replaced with fresh DMEM/F12 containing 1% FCS. The cells were treated with Ang II, ACTH, activin and melatonin or combinations of the reagents at indicated concentrations. After 24-h culture, the medium was removed and total cellular RNA was extracted using TRIzol® (Invitrogen Corp.) and quantified by measuring the absorbance of the sample at 260 nm. Primer pairs for ALK-4, ActRIIA, ActRIIB, MT1, MT2, Smad6, Smad7 and ribosomal protein L19 (RPL19) were selected as we reported previously [8, 22-24]. PCR primer pairs for other target genes were selected from different exons of the corresponding genes to discriminate PCR products that might arise from possible chromosome DNA contaminants as follows: MT1, 19-39 and 366-386 from GenBank accession #NM_005958; MT2, 221-242 and 452-473 from #NM_005959; CYP11B2, 704-723 and 825-844 from #NM_000498; CYP17, 661-681 and 880-900 from #M14564; and ACTH-R/MC2R, 754-773 and 1365-1384 from #X65633. 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 StepOnePlus® real-time PCR system (Applied Biosystems, Foster City, CA) under optimized annealing conditions following the manufacturer's protocol with the following profile: 40 cycles each at 95°C for 3 sec and 60-62°C for 30 sec. The threshold cycle (Ct) values were calculated using StepOnePlus® system software (Applied Biosystems). 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 (1 x 10^5 viable cells/well) were pretreated with indicated concentrations of melatonin and ACTH in serum-free DMEM/F12 in the indicated experiments. After stimulation with activin for 1 to 24 h, 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 an anti-MT1R (H-120) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), an anti-actin antibody (Sigma-Aldrich Co. Ltd.), and anti-phospho-Smad2 (Ser245/250/255) and anti-total-Smad2/3 antibodies (Cell Signaling Technology, Inc., Beverly, MA). The integrated signal density of each protein band was analyzed by the C-DiGit® Blot Scanner System (LI-COR Biosciences, NE). For evaluating MT1 and phospho-Smad2 levels, ratios of the signal intensities of MT1/actin and phospho/total-Smad2 were calculated, respectively.

Statistical analysis

All 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 Fisher's protected least significant difference (PLSD) test or unpaired *t*-test, when appropriate, to determine differences (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). *P* values < 0.05 were accepted as statistically significant.

Results

First, the expression of melatonin receptor MT1 was detected in human adrenocortical H295R cells as well as in normal human adrenal tissue by RT-PCR (**Fig. 1A**), whereas the expression of MT2 was not detected in H295R cells. Treatment with melatonin (10 to 1000 nM) did not affect basal levels of aldosterone or cortisol production (**Fig. 1B,** *upper*) in the medium for 24-h culture, and the ratio of aldosterone to cortisol production was not altered by melatonin (**Fig. 1B,** *lower*). It was revealed that MT1 mRNA (**Fig. 1C**) and MT1 protein (**Fig. 1D**) levels were upregulated by activin (100 ng/ml) for 24 h.

We next examined the effects of melatonin on Ang II- or ACTH-induced aldosterone production. Based on the results of our earlier studies regarding dose- and time-response actions of Ang II and ACTH [6-8], the cells were cultured in a medium containing Ang II (10 nM) or ACTH (100 ng/ml) for 24 h in the presence or absence of activin and melatonin. As shown in **Fig. 2A**, Ang II (10 nM) significantly increased aldosterone (~4 fold) and cortisol (~1.5 fold)

production in the medium for 24-h culture, but treatment with activin alone (100 ng/ml) or in combination with melatonin (30 to 100 nM) had no significant effect on Ang II-induced production of aldosterone. The ratios of aldosterone/cortisol production induced by Ang II were not affected by treatment with activin (100 ng/ml), melatonin (30 to 100 nM), or their combination (**Fig. 2B**).

As shown in **Fig. 3A**, ACTH (100 ng/ml) moderately, but significantly, increased aldosterone production (~1.5 fold) in 24-h cultured medium. In contrast to the effects on Ang II-induced aldosterone synthesis, melatonin treatment (30 nM) significantly augmented ACTH (100 ng/ml)-induced aldosterone synthesis in the presence of activin (100 ng/ml). The ratios of aldosterone/cortisol production were increased by activin effects (100 ng/ml), and the ratios induced by ACTH (100 ng/ml) were also significantly enhanced by melatonin (10 to 100 nM) in combination with activin (100 ng/ml) (**Fig. 3B**). Thus, activin increased ACTH-induced, but not Ang II-induced, aldosterone production. Notably, melatonin enhanced aldosterone production induced by co-treatment with ACTH and activin, although melatonin had no effect on

aldosterone production stimulated by Ang II with activin (**Fig. 2A** and **3A**). These changes in steroidogenesis became apparent when evaluated by the ratios of aldosterone to cortisol (aldosterone/cortisol levels) (**Fig. 2B** and **3B**). Moreover, as shown in **Fig. 3C**, co-treatment with activin and melatonin enhanced aldosterone/cortisol levels stimulated by BtcAMP (0.1 and 1 mM) for 24 h, suggesting that the cAMP-PKA pathway is functionally involved in the upregulation of aldosterone production by activin and melatonin.

In accordance with the results for aldosterone production, ACTH (100 ng/ml) significantly increased cAMP synthesis by H295R cells for 24 h (**Fig. 4A**). Activin (100 ng/ml) tended to increase basal and ACTH-induced cAMP levels, though the effects were statistically insignificant. Of note, melatonin (30 nM) amplified ACTH-induced cAMP synthesis in the presence of activin (100 ng/ml) (**Fig. 4A**), in agreement with the data for aldosterone production induced by ACTH.

Furthermore, the expression levels of aldosterone synthase, CYP11B2, in comparison with 17α-hydroxylase, CYP17, were examined by real-time PCR

analysis. The level of CYP11B2 mRNA was moderately increased by ACTH (100 ng/ml) for 24-h culture, and the ACTH-induced increase in mRNA level was significantly augmented in the condition of co-treatment with activin (100 ng/ml) and melatonin (10 nM) (**Fig. 4B**, *upper*). The level of CYP11B2/CYP17 mRNAs was increased by activin or ACTH treatment, and the ACTH-induced increase was significantly augmented by activin co-treatment (**Fig. 4B**, *lower*). In accordance with the levels of aldosterone/cortisol production, the level of CYP11B2/CYP17 mRNAs stimulated by ACTH and activin was further enhanced in the condition of co-treatment with melatonin (**Fig. 4B**, *lower*).

To determine the mechanism by which melatonin upregulated activin actions in the presence of ACTH, the intensity of intracellular Smad2 signaling induced by activin was evaluated by Western blots. As shown in **Fig. 5A**, Smad2 phosphorylation was readily induced by 1-h stimulation with activin (100 ng/ml). The changes of phosphorylated Smad2 signal intensity induced by activin were quantified by normalizing total Smad2 levels in each treatment. As shown in **Fig. 5A**, activin-induced phospho-Smad2 levels were slightly increased

in the presence of ACTH (100 ng/ml). Of note, in the presence of melatonin action (30 nM), Smad2 phosphorylation induced by activin with ACTH was significantly enhanced (**Fig. 5B**).

To further clarify the mechanism by which melatonin affected ACTH and activin signaling in H295R cells, mRNA levels of key receptors for ACTH and activin pathways were examined by real-time PCR analysis (**Fig. 6**). Melatonin (30 nM) treatment for 24 h had no effect on ACTH-R expression (**Fig. 6A**). The mRNA level of an activin type-I receptor, ALK-4, was significantly increased by melatonin (30 nM) (**Fig. 6B**), while the mRNA levels of type-II receptors including ActRIIA and ActRIIB were not significantly changed by melatonin (**Fig. 6C**). Interestingly, the mRNA levels of inhibitory Smads, Smad6 and Smad7, were significantly reduced by melatonin treatment for 24 h (**Fig. 6D**), suggesting that melatonin facilitates Smad2 activation through downregulating inhibitory Smad6/7.

Thus, it was revealed that melatonin MT1 action enhanced aldosterone production induced by collaborating with ACTH and activin via the cAMP-PKA

pathway (**Fig. 7**). In this mechanism, upregulation of MT1 as well as ALK-4 and downregulation of inhibitory Smad6/7 were functionally linked to the mutual enhancement of MT1 and activin-induced Smad2 signaling in adrenocortical cells.

Discussion

In the present study, we investigated the regulatory effects of melatonin on adrenocortical steroidogenesis using human adrenocortical cells. As shown in **Fig. 7**, melatonin enhanced aldosterone production induced by ACTH and activin via the cAMP-PKA pathway. As for the molecular mechanism, melatonin enhanced activin-induced Smad2 signaling through upregulation of ALK-4 and downregulation of Smad6/7, while activin in turn augmented MT1 expression in adrenocortical H295R cells.

We previously reported the existence of a functional link between ACTH and activin in adrenocortical cells [6], in which activin enhanced ACTH-induced aldosterone synthesis via activation of the cAMP pathway. In addition, ACTH-induced aldosterone synthesis was suppressed by follistatin, which neutralizes activin action by binding [6], suggesting that endogenous activin plays a role in ACTH-induced aldosterone production in this cell line. In the present study, it was revealed that co-treatment with melatonin and activin

enhanced ACTH-induced cAMP synthesis and aldosterone/cortisol levels stimulated by BtcAMP, suggesting that cAMP-PKA signaling is critical for induction of aldosterone production by melatonin and activin in the presence of ACTH.

Activins and inhibins, which also belong to the TGF-β superfamily, are dimeric glycoproteins formed by two of three different subunits including α, βA, and βB. Expression of the activin system in human adrenal tissues and cells has been reported [25, 26]. In those studies, ACTH stimulated the production of inhibins through PKA signaling and decreased the ratio of activin/inhibin, leading to the hypothesis that a functional activin/inhibin system exists within the human adrenal cortex as a local regulator for ACTH signaling. Activin exerts its function by binding to ActRII and subsequent recruitment of ALK-4. In the present study, ALK-4 expression level was significantly increased by melatonin, although melatonin had no effect on ACTH-R expression in H295R cells. Upon binding of BMP or activin ligands to specific type-I and type-II receptors, the receptor complexes cause phosphorylation of intracellular Smads, which then

translocate to the nucleus for regulating target gene transcription. In the present study, activin-induced Smad2 phosphorylation was enhanced, particularly in the presence of both ACTH and melatonin, suggesting functional interaction between melatonin and activin. Upregulation of MT1 and ALK-4 and downregulation of Smad6/7 are likely to be linked to the mutual enhancement of MT1 and activin signaling.

Another major finding in the present experiments was a new functional link between activin and melatonin in the mechanism of ACTH-induced aldosterone production. Transcriptional regulation of the CYP11B2 gene is stimulated by Ang II through AT1R, resulting in an increase in CYP11B2 mRNA and aldosterone production [27, 28]. P450c17, 17α-hydroxylase-C17,20-lyase, is a key enzyme for androgen and corticoid biosynthesis and is encoded by the CYP17A1 gene regulated by ACTH via cAMP [29]. P450c17 is the qualitative regulator of steroidogenesis in human adrenals and, in the presence of this enzymatic activity, glucocorticoids are produced in the zona fasciculata. The ratio of CYP11B2/CYP17 mRNA enabled clarification of aldosterone productivity

induced by ACTH with the results shown as ratios of aldosterone/cortisol synthesis. In accordance with the levels of aldosterone/cortisol production, the levels of CYP11B2/CYP17 mRNA stimulated by ACTH and activin were enhanced in the condition of co-treatment with melatonin.

It has been demonstrated that melatonin inhibits ACTH-stimulated cortisol production in the adrenal gland of capuchin monkeys [17], rats [19] and humans [20] via functional MT1R expressed in the adrenal cortex. Torres-Farfan and colleagues first reported that melatonin inhibited cortisol production induced by ACTH and BtcAMP in dispersed capuchin monkey adrenal cells [17]. The effects were reversed when the cells were co-treated with the MT1/MT2 antagonist luzindole, suggesting the presence of functional melatonin receptors in the adrenal cortex. They also showed by using fetal adrenal gland explants that melatonin selectively inhibited the increase in cortisol production induced by ACTH [18]. Rahman *et al.* reported that melatonin attenuated cortisol secretion induced by forskolin, epinephrine, and ACTH in H295R cells [30]. Campino *et al.* also detected direct inhibitory effects

of melatonin on ACTH-induced responses including cortisol and progesterone production and steroidogenic enzyme expression in the human adrenal gland [20]. It has also been demonstrated by using fetal rats that scheduled melatonin application can entrain adrenal gland rhythms [31].

Based on these findings, we presumed that melatonin has extensive inhibitory effects on adrenal steroidogenesis including aldosterone production. However, in the present study, it was revealed that melatonin rather upregulates aldosterone production induced by ACTH and activin via the cAMP-PKA pathway, which differed from the inhibitory effects on cortisol synthesis. These results suggest a possible crosstalk between melatonin signaling and activin receptor signaling in adrenocortical cells. Concerning the interrelationship between melatonin level and the hypothalamic-pituitary-adrenal axis, it is known that there is an aberrant mode of melatonin secretion in hypercortisolemic conditions such as Cushing's syndrome, implying that excessive cortisol may disturb the normal melatonin rhythm and *vice versa*.

It has been, in general, recognized that H295R cells have a weak ACTH

response possibly due to a low expression level of ACTH-R [32, 33] despite the detectable expression of ACTH-R [34]. In this regard, we have reported that ACTH induces cAMP synthesis, resulting in cortisol and aldosterone production in H295R cells [6]. In other studies on H295R cells, Janes *et al.* [35] revealed that ACTH induces a transient ERK1/2 response via ACTH-R and Lucki *et al.* [36] showed that ACTH induces StAR expression. A steroid profiling revealed that H295R cells in a steady condition secrete cortisol predominantly, while aldosterone and other steroids are secreted at much lower levels [37]. On the other hand, it is notable that aldosterone output can be specifically activated in response to various stimuli such as ACTH and angiotensin II and, in particular, to potassium [37], suggesting characteristics favorable to an *in vitro* model of hyperaldosteronism [37]. By utilizing this feature of H295R cells, it was uncovered that ACTH-induced cAMP-PKA activity is likely to be a key for upregulation of aldosterone production caused by activin and melatonin.

There has been an accumulation of reports regarding the expressional and functional relationship between melatonin and TGF-β. For instance, it has

been shown that melatonin increases TGF-β synthesis in human prostate epithelial cells, leading to melatonin-mediated attenuation of cell proliferation [38]. Inhibition of breast cancer cell proliferation by melatonin with vitamin D3 seems to be linked to activation of Smads [39]. Melatonin activates an osteogenic process by promoting the expression of BMP-2 and -4 via ERK and Wnt pathways [40]. We have also reported the effect of melatonin on regulation of ACTH production by corticotrope cells, in which MT1 and BMP-4 actions were mutually enhanced [23]. In ovarian granulosa cells, melatonin was shown to suppress BMP-6-induced Smad1/5/8 signaling by reducing Smad6 expression [24]. The BMP/activin system is a fine regulator of endocrine activity at various levels including the adrenal gland. Further research is needed to conclude whether melatonin is a functional clue for the integration of systemic aldosterone/cortisol balance in adrenal steroidogenesis.

Collectively, the results showed that melatonin facilitated aldosterone production induced by ACTH combined with activin via the cAMP-PKA pathway (**Fig. 7**). The results also suggested a mutual interaction between melatonin

and activin receptor signaling for regulating ACTH-dependent aldosterone synthesis in the adrenal. In contrast to the suppressive effect of melatonin on cortisol production [17, 20], melatonin appeared to comparably facilitate the induction of aldosterone output in the presence of ACTH and activin. This finding may lead to a new strategy for the modulation of mineral/water balance and blood pressure by controlling systemic aldosterone/cortisol levels with utilization of adrenocortical melatonin activity.

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Figure Legends:

Fig. 1. Expression of melatonin receptors and effect of melatonin on aldosterone synthesis in human adrenocortical cells. **A**) Expression of mRNAs encoding MT1 (368 bp) and RPL19 (190 bp) was examined by RT-PCR analysis in H295R cells compared with that in normal human adrenal tissue. MM indicates molecular weight marker. **B**) After cells (3 x 10^5 cells/well) had been precultured in 24-well plates with 10% FCS, the medium was changed to DMEM/F12 containing 1% FCS, and then the cells were treated with indicated concentrations of melatonin. After 24-h culture, aldosterone concentrations and ratios of aldosterone/cortisol production in the culture media were determined. **C**) Total cellular RNA was extracted from H295R cells that had been treated with activin in DMEM/F12 containing 1% FCS for 24 h, and mRNA levels of MT1 were determined by quantitative PCR. The mRNA levels of MT1/RPL19 were expressed as fold changes. **D**) After preculture in a serum-free condition, cells (1 x 10^5 viable cells/well) were treated with activin for 24 h. The cell lysates were then subjected to SDS-PAGE/immunoblotting

analysis using anti-MT1 and anti-actin antibodies. The integrated signal density of each protein band was digitally analyzed, and the ratios of signal intensities of MT1/actin were calculated. Results are shown as means ± SEM. The results were analyzed by ANOVA (**B**) and unpaired *t*-test (**C, D**). *, $P < 0.05$ vs. control group.

Fig. 2. Effects of melatonin and activin on Ang II-induced aldosterone production by human adrenocortical cells. After cells (3 x 10^5 cells/well) had been precultured in 24-well plates with 10% FCS, the medium was changed to DMEM/F12 containing 1% FCS, and then the cells were treated with indicated concentrations of Ang II, activin and melatonin. After 24-h culture, **A**) aldosterone and **B**) cortisol concentrations and **C**) ratios of aldosterone/cortisol production in the culture media were determined. Results are shown as means ± SEM. The results were analyzed by ANOVA. For results within a panel, the values with different superscript letters are significantly different at $P < 0.05$.

Fig. 3. Effects of melatonin and activin on ACTH- and BtcAMP-induced aldosterone production by human adrenocortical cells. After cells (3 x 10^5 cells/well) had been precultured in 24-well plates with 10% FCS, the medium was changed to DMEM/F12 containing 1% FCS, and then the cells were treated with indicated concentrations of activin and melatonin in the presence of **A**, **B**) ACTH or **C**) BtcAMP. After 24-h culture, **A**) aldosterone concentrations and **B**, **C**) ratios of aldosterone/cortisol production in the culture media were determined. Results are shown as means ± SEM. The results were analyzed by the unpaired *t*-test (**A**, **B**) and ANOVA (**C**). *, $P < 0.05$ and **, $P < 0.01$ vs. control group or between the indicated groups.

Fig. 4. Effects of melatonin and activin on ACTH-induced cAMP synthesis and steroidogenetic enzyme expression by human adrenocortical cells. **A**) After cells (3 x 10^5 cells/well) had been precultured in 24-well plates with 10% FCS, the medium was changed to DMEM/F12 containing 1% FCS containing IBMX, and then the cells were treated with indicated concentrations of ACTH,

activin and melatonin. After 24-h culture, cAMP concentrations were determined in the culture media. **B**) Total cellular RNA was extracted from H295R cells that had been treated with ACTH, activin and melatonin in DMEM/F12 containing 1% FCS for 24 h, and mRNA levels of CYP11B2 and CYP17 were determined by quantitative PCR. The mRNA levels of CYP11B2/RPL19 and CYP11B2/CYP17 were expressed as fold changes. Results are shown as means ± SEM. The results were analyzed by the unpaired *t*-test (**A**) and ANOVA (**B**). *, $P < 0.05$ and **, $P < 0.01$ vs. control group or between the indicated groups.

Fig. 5. Effects of melatonin and ACTH on activin-induced Smad2 phosphorylation in human adrenocortical cells. After preculture in serum-free conditions with melatonin and ACTH, cells (1 x 10^5 viable cells/well) were treated with activin for 60 min. **A**) The cell lysates were then subjected to SDS-PAGE/immunoblotting analysis using anti-pSmad2 and anti-tSmad2/3 antibodies. **B**) The integrated signal density of each protein band was digitally

analyzed, and the ratios of signal intensities of pSmad2/tSmad2 were calculated. Results are shown as means ± SEM. The results were analyzed by ANOVA. The values with different superscript letters are significantly different at $P < 0.05$ (**B**).

Fig. 6. Effects of melatonin on expression of the ACTH receptor, BMP receptors and Smad6/7 in human adrenocortical cells. Total cellular RNA was extracted from H295R cells treated with melatonin in DMEM/F12 containing 1% FCS for 24 h, and **A**) ACTH-R, **B**) ALK-4, **C**) ActRIIA and ActRIIB, and **D**) Smad6 and Smad7 were determined by quantitative PCR. The expression levels of target gene mRNA were standardized by RPL19 level in each sample, and then levels of mRNA of target genes were expressed as fold changes. Results are shown as means ± SEM. The results were analyzed by the unpaired *t*-test. *, $P < 0.05$ vs. control groups.

Fig. 7. Interaction of melatonin and activin in ACTH-induced aldosterone

production by human adrenocortical cells. Melatonin enhances aldosterone synthesis induced by ACTH and activin via the cAMP-PKA pathway, through relative increase of CYP11B2 expression by adrenocortical cells. The effects of melatonin MT1 causing upregulation of ALK-4 and downregulation of inhibitory Smad6/7 and the effect of activin on MT1 expression are involved in the activin-induced Smad2 activation, leading to enhancement of aldosterone production.

Fig. 1

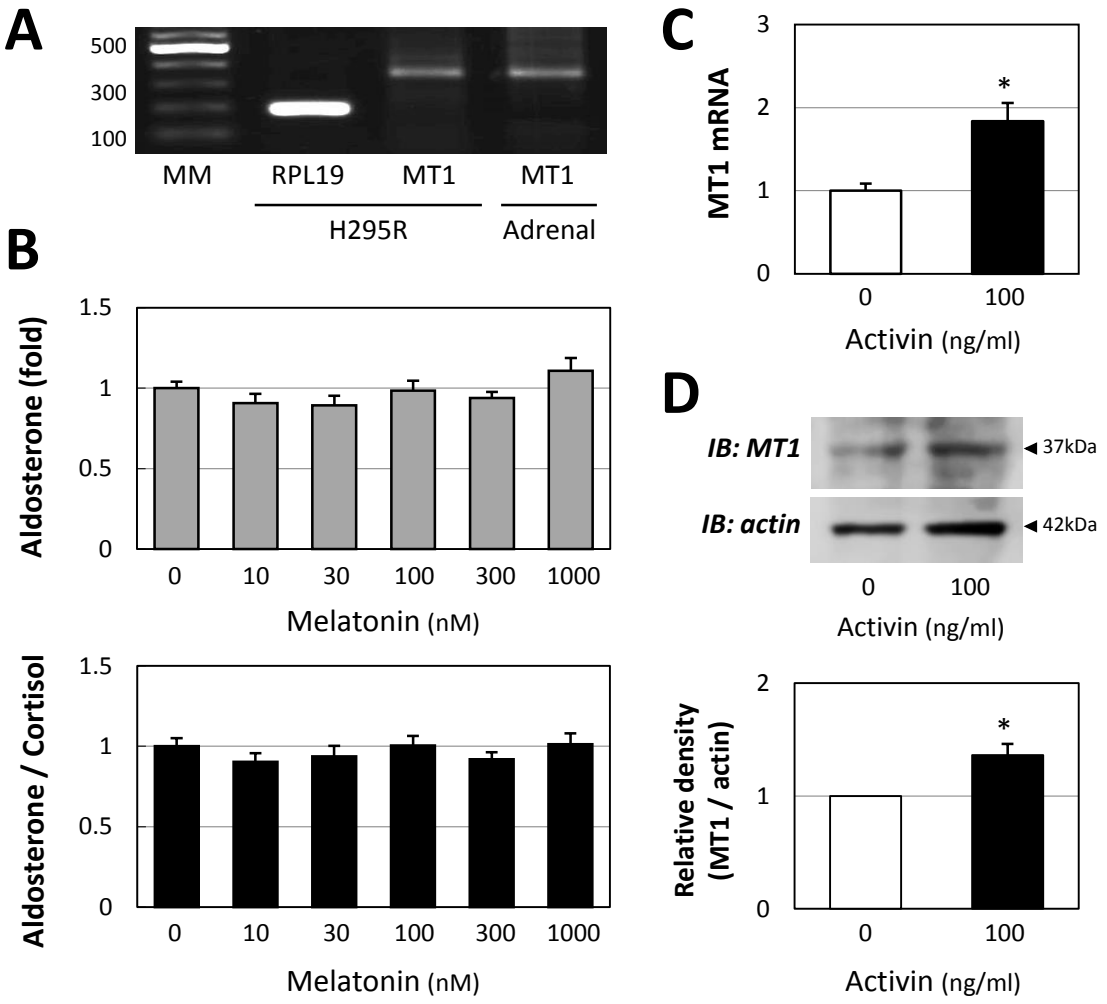

A
500
300
100
MM
RPL19
MT1
MT1
H295R
Adrenal
B
Aldosterone (fold)
1.5
1
0.5
0
0
10
30
100
300
1000
Melatonin (nM)
Aldosterone / Cortisol
1.5
1
0.5
0
0
10
30
100
300
1000
Melatonin (nM)
C
MT1 mRNA
3
2
1
0
*
0
100
Activin (ng/ml)
D
IB: MT1
37kDa
IB: actin
42kDa
0
100
Activin (ng/ml)
Relative density (MT1 / actin)
2
1
0
*
0
100
Activin (ng/ml)

Fig. 2

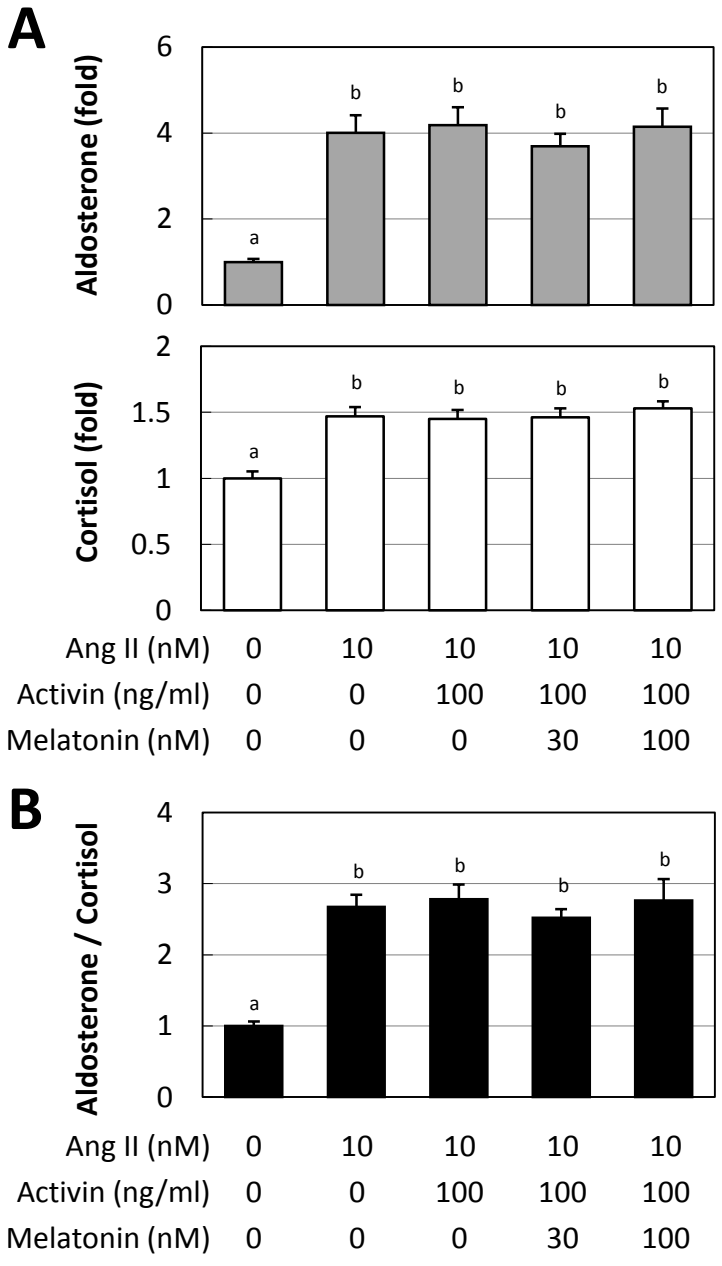

A
Aldosterone (fold)
6
4
2
0
a
b
b
b
b
Cortisol (fold)
2
1.5
1
0.5
0
a
b
b
b
b
Ang II (nM) 0 10 10 10 10
Activin (ng/ml) 0 0 100 100 100
Melatonin (nM) 0 0 0 30 100
B
Aldosterone / Cortisol
4
3
2
1
0
a
b
b
b
b
Ang II (nM) 0 10 10 10 10
Activin (ng/ml) 0 0 100 100 100
Melatonin (nM) 0 0 0 30 100

Fig. 3

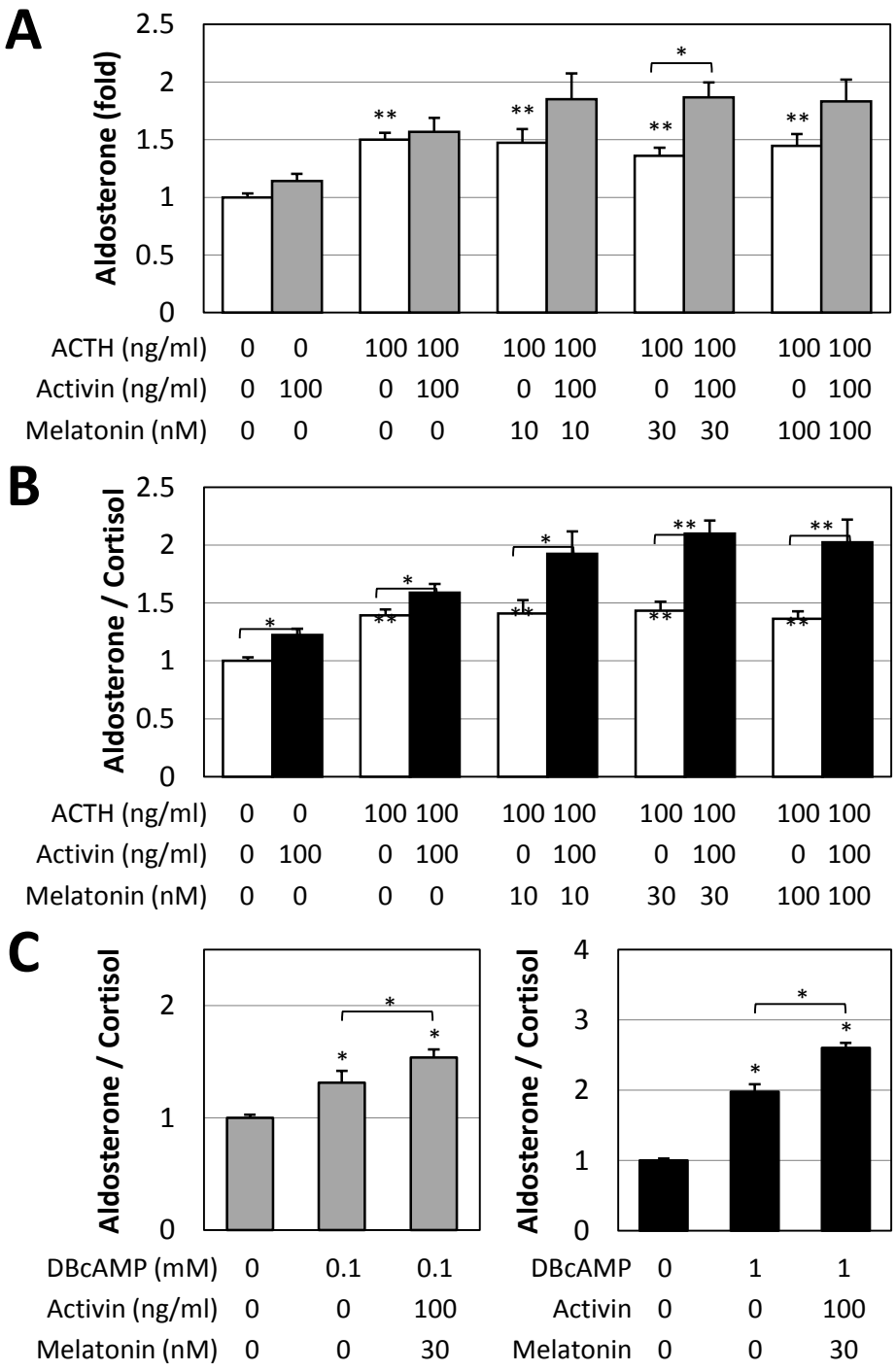

A
Aldosterone (fold)
2.5
2
1.5
1
0.5
0
ACTH (ng/ml) 0 0 100 100 100 100 100 100 100 100
Activin (ng/ml) 0 100 0 100 0 100 0 100 0 100
Melatonin (nM) 0 0 0 0 10 10 30 30 100 100
B
Aldosterone / Cortisol
2.5
2
1.5
1
0.5
0
ACTH (ng/ml) 0 0 100 100 100 100 100 100 100 100
Activin (ng/ml) 0 100 0 100 0 100 0 100 0 100
Melatonin (nM) 0 0 0 0 10 10 30 30 100 100
C
Aldosterone / Cortisol
2
1
0
DBcAMP (mM) 0 0.1 0.1
Activin (ng/ml) 0 0 100
Melatonin (nM) 0 0 30
Aldosterone / Cortisol
4
3
2
1
0
DBcAMP 0 1 1
Activin 0 0 100
Melatonin 0 0 30

Fig. 4

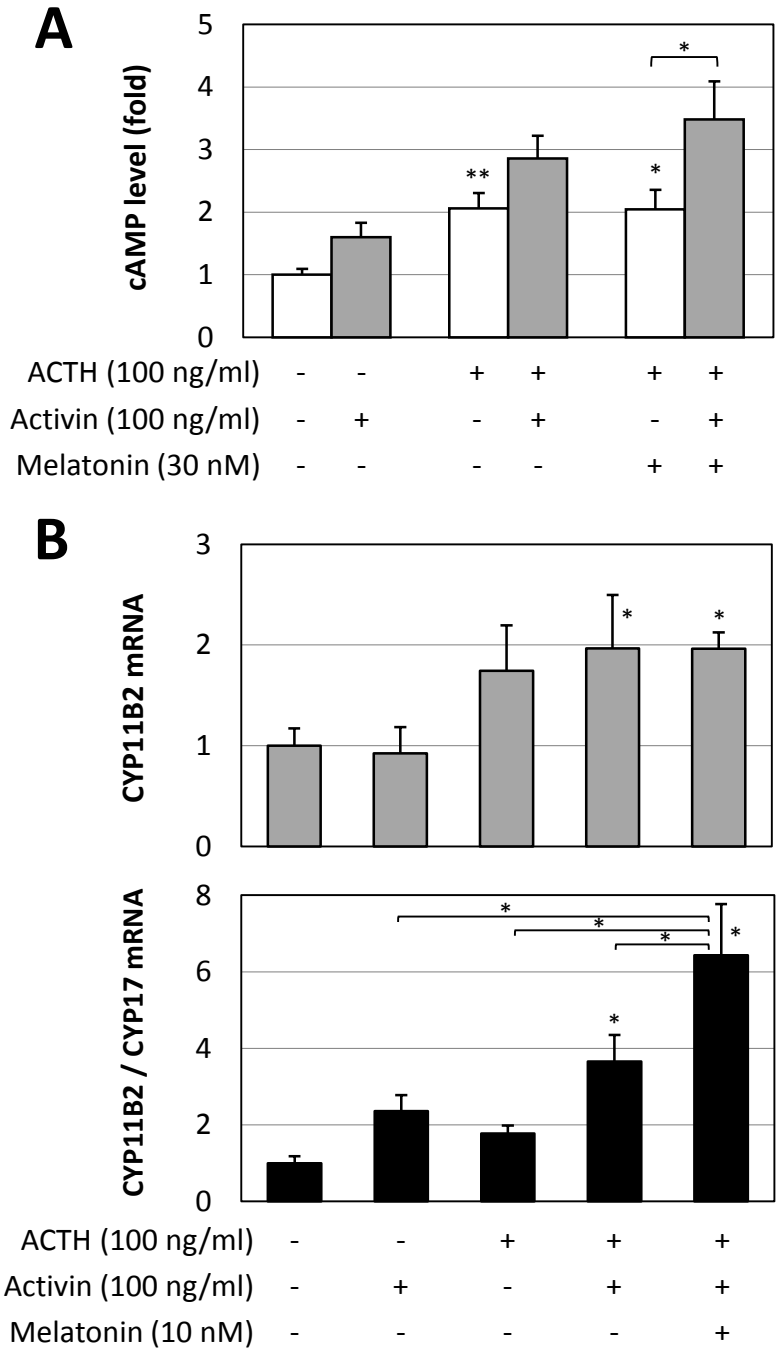

A
cAMP level (fold)
5
4
3
2
1
0
*
**
*
ACTH (100 ng/ml) - - + + + +
Activin (100 ng/ml) - + - + - +
Melatonin (30 nM) - - - - + +
B
CYP11B2 mRNA
3
2
1
0
*
*
CYP11B2 / CYP17 mRNA
8
6
4
2
0
*
*
*
*
*
ACTH (100 ng/ml) - - + + +
Activin (100 ng/ml) - + - + +
Melatonin (10 nM) - - - - +

Fig. 5

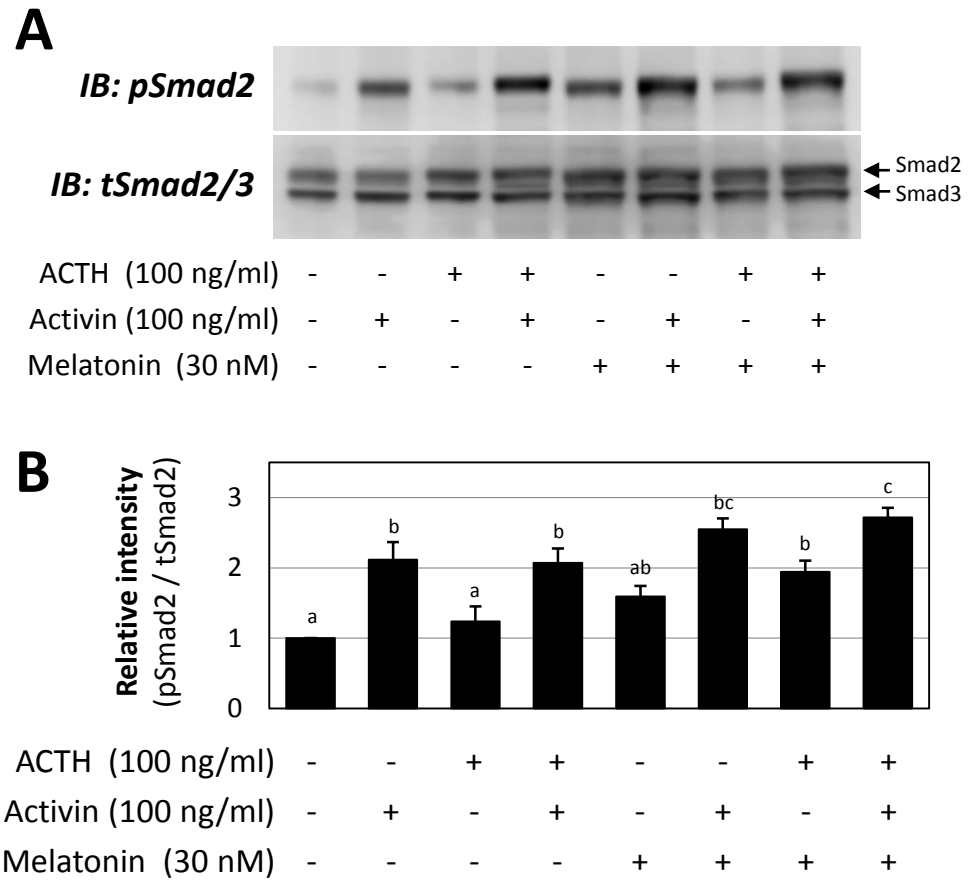

A
IB: pSmad2
IB: tSmad2/3
Smad2
Smad3
ACTH (100 ng/ml) - - + + - - + +
Activin (100 ng/ml) - + - + - + - +
Melatonin (30 nM) - - - - + + + +
B
Relative intensity
(pSmad2 / tSmad2)
0
1
2
3
a
b
a
b
ab
bc
b
c
ACTH (100 ng/ml) - - + + - - + +
Activin (100 ng/ml) - + - + - + - +
Melatonin (30 nM) - - - - + + + +

Fig. 6

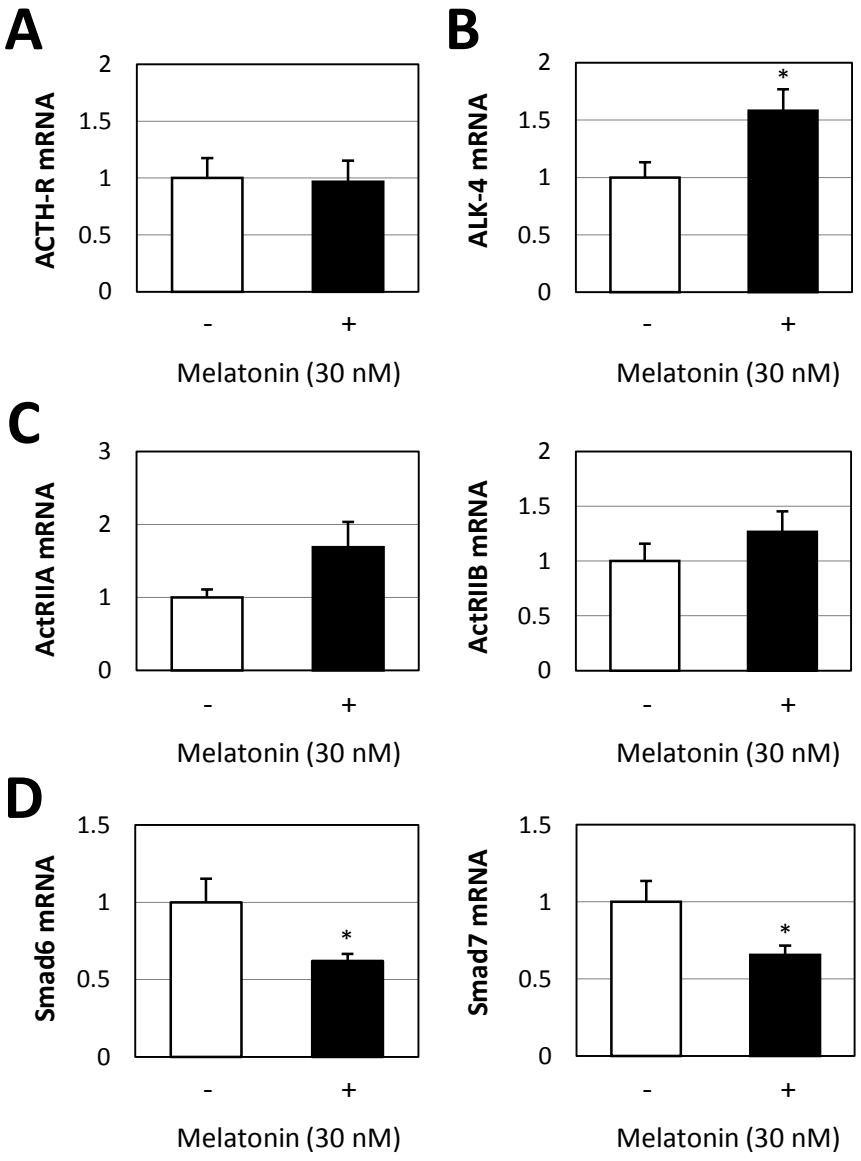
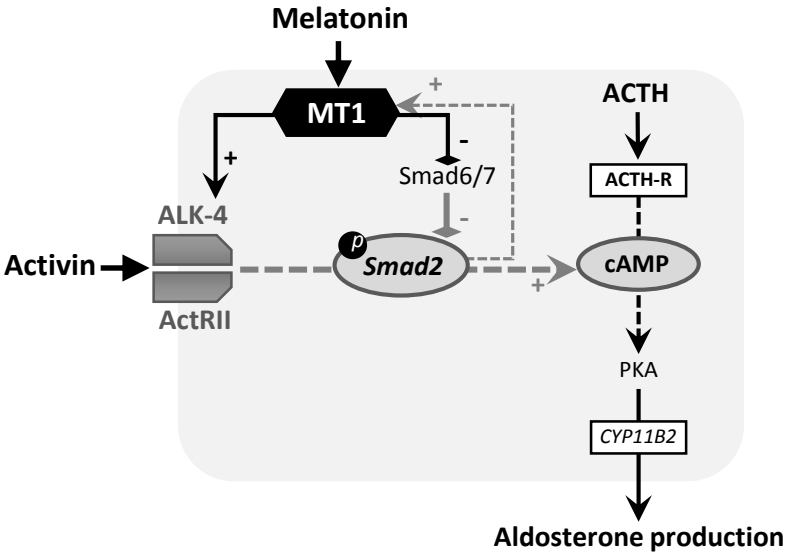

A
ACTH-R mRNA
2
1.5
1
0.5
0
-
+
Melatonin (30 nM)
B
ALK-4 mRNA
*
2
1.5
1
0.5
0
-
+
Melatonin (30 nM)
C
ActRIIA mRNA
3
2
1
0
-
+
Melatonin (30 nM)
ActRIIB mRNA
2
1.5
1
0.5
0
-
+
Melatonin (30 nM)
D
Smad6 mRNA
*
1.5
1
0.5
0
-
+
Melatonin (30 nM)
Smad7 mRNA
*
1.5
1
0.5
0
-
+
Melatonin (30 nM)

Fig. 7


Melatonin
MT1
+
-
+
Smad6/7
-
ALK-4
Activin
ActRII
P
Smad2
+
ACTH
ACTH-R
cAMP
PKA
CYP11B2
Aldosterone production