Melatonin regulates catecholamine biosynthesis by modulating bone morphogenetic protein and glucocorticoid actions.

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Abbreviations:

ActRI, activin type-I receptor	ActRII, activin type-II receptor
ALK, activin receptor-like kinase	AR, androgen receptor
BMP, bone morphogenetic protein	BMPRI, BMP type-I receptor
BMPRII, BMP type-II receptor	DBH, dopamine-β-hydroxylase
DDC, 3,4-dihydroxyphenylalanine decarboxylase	
DHT, dihydrotestosterone	GR, glucocorticoid receptor
MT, melatonin receptor	MR, mineralocorticoid receptor
PKA, protein kinase A	PNMT, phenylalanine N-methyltrasferase
TGF, transforming growth factor	TH, tyrosine hydroxylase

Abstract

Melatonin is functionally involved in the control of circadian rhythm and hormonal secretion. In the present study, we investigated the roles of melatonin in the interaction of catecholamine synthesis with adrenocortical steroids by focusing on bone morphogenetic protein (BMP)-4 expressed in the adrenal medulla using rat pheochromocytoma PC12 cells. Melatonin treatment significantly reduced the mRNA expression of catecholamine synthases, including the rate-limiting enzyme tyrosine hydroxylase (*Th*), 3,4-dihydroxyphenylalanine decarboxylase and dopamine-β-hydroxylase expressed in PC12 cells. In accordance with changes in the expression levels of enzymes, dopamine production and cAMP synthesis determined in the culture medium and cell lysate were also suppressed by melatonin. The MT1 receptor, but not the MT2 receptor, was expressed in PC12 cells, and luzindole treatment reversed the inhibitory effect of melatonin on *Th* expression, suggesting that MT1 is a functional receptor for the control of catecholamine synthesis.

Interestingly, melatonin enhanced the inhibitory effect of BMP-4 on *Th* mRNA expression in PC12 cells. Melatonin treatment accelerated BMP-4-induced phosphorylation of SMAD1/5/8 and transcription of the BMP target gene *Id1*. Of note, melatonin significantly upregulated *Alk2* and *Bmpr2* mRNA levels but suppressed inhibitory *Smad6/7* expression, leading to the enhancement of SMAD1/5/8 signaling in PC12 cells, while BMP-4 did not affect *Mt1* expression. Regarding the interaction with adrenocortical steroids, melatonin preferentially enhanced glucocorticoid-induced *Th* mRNA through upregulation of the glucocorticoid receptor and downregulation of *Bmp4* expression, whereas melatonin repressed *Th* mRNA expression induced by aldosterone or androgen without affecting expression levels of the receptors for mineralocorticoid and androgen. Collectively, the results indicate that melatonin plays a modulatory role in catecholamine synthesis by cooperating with BMP-4 and glucocorticoid in the adrenal medulla.

Introduction

The adrenal cortex and medullar tissues functionally interact in a paracrine manner through their close anatomical structures [1, 2]. Endogenous glucocorticoids promote catecholamine synthesis by inducing catecholamine-synthesizing enzymes via the cortico-medullary portal system in the adrenal [3]. We earlier reported the presence of a bone morphogenetic protein (BMP) system consisting of type-I and -II receptors in adrenomedullary cells [4, 5] as well as 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 steroidogenesis [10-12]. For instance, BMP-6 is involved in the maintenance of angiotensin II-induced aldosterone production by upregulating MAPK signaling [7-9], while the activin system is linked to the adrenocorticotropin-induced cAMP-protein kinase A (PKA) pathway in adrenocortical cells [6, 13]. Endogenous BMP-4 and its signaling machinery are expressed in the adrenal medulla and they play a

regulatory role in catecholamine synthesis induced by adrenocortical steroids [4, 5].

On the other hand, melatonin plays crucial roles in the physiologically circadian and seasonal rhythms and in the functions of hormones and cytokines [14-16]. Melatonin actions are elicited via G protein-coupled MT1 and MT2 receptors expressed in the brain and various peripheral tissues. Melatonin receptors, mainly the MT1 receptor, have been detected in adrenal tissues and cells in mammals [17-20]. As for the effects of melatonin on adrenocortical hormones, it has been reported that melatonin, directly on the adrenal gland, inhibits glucocorticoid synthesis in response to adrenocorticotropin (ACTH) in various species [18, 21-23]. Melatonin exerts inhibitory effects on ACTH secretion in the anterior pituitary and cortisol production in the adrenal by different mechanisms. We recently reported that melatonin suppresses ACTH secretion via BMP-4 action in corticotrope cells [24]. It was also found that, under the influence of ACTH, melatonin facilitates aldosterone production by cooperating with activin in adrenocortical cells [13]. Interestingly, melatonin

has been reported to suppress nocturnal blood pressure [25, 26]. In a clinical study, oral administration of melatonin significantly reduced blood pressure, the pulsatility index in the internal carotid artery, and levels of catecholamines [27], suggesting that melatonin may reduce contractile tone and activity of the cardiovascular system by regulating blood pressure and catecholamine levels.

However, the effects of melatonin on adrenomedullary functions have yet to be elucidated. Here we investigated roles of melatonin in catecholamine synthesis by focusing on BMP-4 expressed in the adrenal medulla and on the functional interaction with adrenocortical steroids using rat pheochromocytoma cells. It was revealed that melatonin plays a modulatory role in catecholamine synthesis by cooperating with BMP-4 activity and glucocorticoid effects in the adrenal medulla.

Materials and Methods

Reagents and supplies

Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin solution, and d-aldosterone, dexamethasone, dihydrotestosterone (DHT), 3-isobutyl-1-methylxanthine (IBMX), melatonin, luzindole, fetal calf serum albumin and horse serum albumin were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Recombinant human BMP-4 was purchased from R&D Systems (Minneapolis, MN). Normal rat adrenal tissues were obtained from male Sprague-Dawley (SD) rats (Charles River Laboratories, Wilmington, MA). The animal protocols were approved by Okayama University Institutional Animal Care and Use Committee.

Cell culture

The rat adrenal medullary cell line PC12 was obtained from RIKEN Cell Bank (Tsukuba, Ibaraki, Japan). PC12 cells were maintained in DMEM

supplemented with 10% fetal calf serum (FCS), 10% horse serum (HS), penicillin, and streptomycin at 37°C in a 5% CO2 humidified atmosphere. The culture medium was changed twice/week, and cultures were passaged at ~80% confluence.

Catecholamine assays

PC12 cells (3 x 10^5 viable cells/well) were precultured in 12-well plates with DMEM containing 10% FCS and 10% HS for 24 h. The medium was then changed to DMEM containing 1% FCS and 1% HS, and the cells were treated with indicated concentrations of melatonin. The culture medium was collected after 24-h culture, and the levels of catecholamines, including dopamine, noradrenaline and adrenaline, were determined by high-performance liquid chromatography (HPLC; BML, Inc., Saitama, Japan).

Measurement of cAMP production

To assess cellular cAMP synthesis, cells (3 x 10^5 viable cells/well) were

precultured in 12-well plates with DMEM containing 10% FCS and 10% HS for 24 h. The medium was then changed to DMEM containing 1% FCS, 1% HS and 0.1 mM IBMX (a phoshodiesterase inhibitor) and the cells were treated with indicated concentrations of melatonin. After 24-h culture, the medium and cell lysates solubilized with 0.1 m HCl were collected, and the extracellular and intracellular 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 (3 x 10^5 viable cells/well) were precultured in 12-well plates with DMEM containing 10% FCS and 10% HS for 24 h. The medium was then replaced with DMEM containing 1% FCS and 1% HS, and the cells were treated with melatonin, luzindole, BMP-4, aldosterone, dexamethasone, DHT, or combinations of the reagents at the 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. Total RNA of adrenal tissues was also extracted using TRIzol® from male SD rats. 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, 145–165 and 357–377 (from GenBank accession #NM_008639); MT2, 214–235 and 445–466 (NM_145712); androgen receptor (AR), 2521-2543 and 2711-2734 (NM_013476); and glucocorticoid receptor (GR), 51-75 and 180-200 (M14053). Primer pairs for catecholamine synthase genes, including tyrosine hydroxylase (*Th*), 3,4-dihydroxyphenylalanine decarboxylase (*Ddc*) and dopamine-β-hydroxylase (*Dbh*), mineralocorticoid receptor (*Mr*), *Id1*, *Bmp4*, *Alk2*, *Alk3*, *Bmpr2*, *Smad6*, *Smad7*, and ribosomal protein L19 (*Rpl19*) were selected as we reported previously [4, 5, 28, 29]. 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 GmbH) under optimized annealing conditions following 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 GmbH). 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

PC12 cells (1 x 10^5 viable cells/well) were pretreated with the indicated concentrations of melatonin in serum-free DMEM in the indicated experiments.

Cells were treated with melatonin or dexamethasone for 24 h. In the indicated experiments, cells were stimulated by BMP-4 for 1 h after the treatment with melatonin. 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 subjected to SDS-PAGE/immunoblotting analysis using an anti-MT1 (R-18) antibody, an anti-MT2 (T-18) antibody, an anti-GR (M-20) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), an antiphospho-SMAD1/5/8 antibody (Cell Signaling Technology, Inc., Beverly, MA), and an 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 GR levels, ratios of the signal intensities of GR/actin were calculated.

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

Effects of melatonin on catecholamine synthesis by rat adrenomedullar cells.

Expression of the melatonin receptor *Mt1* was detected in rat pheochromocytoma PC12 cells as well as in normal rat whole adrenal tissue and adrenal medullary tissue by RT-PCR (**Fig. 1A**), whereas *Mt2* expression was not detected. MT1 protein expression was also detected by Western blots in PC12 cells (**Fig. 1B**) regardless of the conditions of treatment with melatonin (100 nM) and dexamethasone (100 nM). On the other hand, MT2 receptor expression was not detected either by PCR or Western blots (data not shown).

Based on the results of our earlier studies [5], PC12 cells predominantly secrete dopamine, but the levels of adrenaline and noradrenaline are undetectable. Mean dopamine levels of the control groups were 185 pg/ml and 20.3 ng/well in the medium and in the cell lysate, respectively. Melatonin suppressed dopamine secretion in the medium and intracellular dopamine

production in the cell lysate by PC12 cells (**Fig. 1C**). The mRNA levels of catecholamine synthases, including *Th, Ddc and Dbh*, were also determined by quantitative real-time PCR analysis. As shown **Fig. 1D**, the levels of *Th, Ddc and Dbh* mRNA were decreased by melatonin treatment (0.03 to 3 μM) in a concentration-dependent manner.

Cells were treated with melatonin (100 to 1000 nM) in the presence or absence of the MT1/MT2 antagonist luzindole (10 to 100 nM) for 24 h. Treatment with luzindole alone did not affect the basal *Th* mRNA expression, indicating that the levels of endogenous melatonin synthesis are negligible (**Fig. 1E**). Treatment with luzindole reversed the inhibitory effects of melatonin on *Th* mRNA expression in a concentration-responsive manner, suggesting that the effects of melatonin are mediated via MT1 expressed on PC12 cells (**Fig. 1E**). In accordance with the results for dopamine concentration and its synthetic enzyme expression, cAMP synthesis in the medium and intracellular levels were also decreased by melatonin treatment (0.1 to 1 μM) (**Fig. 1F**).

Interaction of melatonin and BMP-4 in the regulation of catecholamine synthesis.

Cells were cultured in the presence or absence of melatonin (100 nM) and BMP-4 (10 ng/ml) for 24 h, and the levels of TH, a rate-limiting enzyme of catecholamine synthesis, were determined by quantitative real-time PCR analysis. As shown in **Fig. 2A**, treatments with melatonin and BMP-4 contributed to a decrease in *Th* mRNA levels, and co-treatment with melatonin and BMP-4 enhanced the reduction of *Th* mRNA. As shown in **Fig. 2B**, SMAD1/5/8 phosphorylation was readily induced by 1-h stimulation with BMP-4 (10 ng/ml), and the BMP-4-induced phosphorylation of SMAD1/5/8 was enhanced by pretreatment with melatonin (100 nM). The mRNA levels of *Id1*, one of the direct BMP target genes, were also increased by co-treatment with melatonin (100 nM) and BMP-4 (10 ng/ml) (**Fig. 2C**).

To clarify the mechanism by which melatonin upregulated BMP-4 action, mRNA levels of *Mt1* and receptors for BMP-4 pathways were examined by quantitative real-time PCR analysis. BMP-4 (10 ng/ml) treatment had no effect

on *Mt1* expression, while melatonin (100 nM) treatment increased the expression of endogenous *Bmp4* mRNA for 24 h (**Fig. 2D**). Moreover, mRNA levels of the BMP type-I receptor *Alk2* and the type-II receptor *Bmpr2* were significantly increased by melatonin (100 nM), while *Alk3* expression was not significantly changed by melatonin (**Fig. 2E**). Interestingly, mRNA levels of inhibitory *Smad6* and *Smad7* were reduced by melatonin treatment for 24 h, suggesting that melatonin facilitates SMAD1/5/8 activation through downregulating inhibitory *Smad6/7* in PC12 cells (**Fig. 2E**).

Interaction of melatonin and adrenocortical steroids in catecholamine biosynthesis.

As shown in **Fig. 3A**, *Th* mRNA levels were significantly increased by independent treatments with aldosterone (100 nM), dexamethasone (100 nM) and DHT (100 nM) for 24 h. Addition of melatonin (0.1 to 1 μM) suppressed *Th* mRNA expression induced by aldosterone and DHT. On the other hand, melatonin treatment significantly enhanced *Th* mRNA expression induced by

dexamethasone in a concentration-responsive manner (**Fig. 3A**).

Expression of *Mr, Gr and Ar* was detected in PC12 cells (**Fig. 3B**). Of note, *Gr* mRNA level was significantly increased by melatonin treatment for 24 h, whereas melatonin had no effect on mRNA levels of *Mr and Ar* in PC12 cells (**Fig. 3C**). The levels of GR protein expression were also concomitantly increased by treatment with melatonin (100 nM) (**Fig. 3D**). In addition, *Bmp4* mRNA levels were decreased by melatonin (100 nM) treatment in the presence of dexamethasone (100 nM) (**Fig. 3E**). These results suggested that upregulation of *Gr* as well as downregulation of *Bmp4* induced by melatonin may have contributed to the enhancement of *Th* induction caused by co-treatment with glucocorticoid and melatonin.

Discussion

In the present study, it was shown that melatonin contributed to the regulation of catecholamine synthesis in cooperation with BMP-4 activity and glucocorticoid effects. As shown in **Fig. 4**, melatonin suppressed catecholamine production via the cAMP-PKA pathway, and melatonin and BMP-4 coordinately reduced catecholamine production. As for the molecular mechanism, melatonin enhanced *Bmp4* expression and BMP-4-induced SMAD1/5/8 signaling through upregulation of *Alk2* and *Bmpr2* and downregulation of *Smad6/7* in rat adrenomedullary cells.

Regarding the receptors for melatonin action, MT1, but not MT2, subtype was expressed in PC12 cells. The expression of MT1 has been demonstrated in adrenal tissues and cells of rats, humans and primates [17-20, 22], whereas the expression of MT2 was found by RT-PCR only in a study using rat adrenal tissue [17]. Treatment with the MT1/MT2 antagonist luzindole alone did not affect basal *Th* mRNA expression, indicating that the levels of

endogenous melatonin production in PC12 cells are negligible. Given that luzindole significantly reversed the inhibitory effects of melatonin on *Th* mRNA expression, it is thought that the effects of melatonin are mediated via MT1 receptors expressed on PC12 cells.

We previously reported a regulatory role of BMP-4 in catecholamine synthesis by adrenomedullary cells [4, 5], in which BMP-4 enhanced catecholamine synthesis induced by adrenocortical steroids. In the present study, it was revealed that co-treatment with melatonin and BMP-4 cooperatively suppressed catecholamine biosynthesis. *Alk2* and *Bmpr2* expression levels were significantly increased by melatonin, though BMP-4 had no effect on *Mt1* expression in PC12 cells. Upon binding of BMP 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. Of note, melatonin enhanced SMAD1/5/8 phosphorylation and *Id1* mRNA expression induced by BMP-4, suggesting a functional interaction between melatonin and BMP-4. Downregulation of *Smad6*/7 by melatonin is

also likely to be linked to the enhancement of BMP signaling. Based on these findings, BMP-4 might play a physiological role in the regulation of catecholamine production in the adrenal medulla.

Regarding the possible mechanisms by which melatonin directly suppresses catecholamine induction, it has been reported that a high concentration of melatonin inhibits the voltage-sensitive calcium-channel of PC12 cells in a melatonin receptor-independent manner without cAMP modulation by inhibiting neurotransmitter release [30], suggesting modulation of neurotransmitter release as a possible cellular mechanism for the antiepileptic effect of melatonin. It has also been reported that melatonin inhibits nicotine-stimulated dopamine release [31]. Chronic exposure to pertussis toxin diminishes the inhibitory effect of melatonin on nicotine-invoked dopamine response, suggesting that this inhibition is partially mediated by the pertussis toxin-sensitive G-protein of the melatonin receptor.

In the present study, the existence of a functional interaction between melatonin and glucocorticoids was also uncovered in the adrenal medulla.

Regarding the influence of melatonin on regulation of systemic steroids, it has been reported that melatonin inhibits the glucocorticoid response to ACTH in various animal models [18, 21-23]. Melatonin also directly antagonizes GR-mediated effects such as GR-induced apoptosis in thymocytes [32]. Interestingly, GR action, in turn, activates catecholamine-induced melatonin production through inhibition of NF-kB translocation in the pineal gland [33]. Based on these findings, we presumed that melatonin would inhibit glucocorticoid-induced catecholamine production. However, in our study using PC12 cells, melatonin actually enhanced glucocorticoid-induced catecholamine synthesis with upregulation of GR expression and downregulation of *Bmp4* expression in the presence of a glucocorticoid, whereas melatonin suppressed the production of catecholamine induced by aldosterone and androgen without affecting *Mr* and *Ar* expression. It is likely that melatonin exerts unique modulatory effects on GR activities depending on the cells or tissues expressing GR, though it remains to be determined whether the melatonin-induced GR upregulation is cell-specific or not. Thus, the activities of catecholamines,

glucocorticoids and melatonin are mutually regulated at hormone-producing sites.

A functional interaction between melatonin and TGF-β molecules has also been reported. For instance, melatonin increases TGF-β synthesis in human prostate epithelial cells [34], leading to melatonin-mediated attenuation of cell proliferation. Inhibition by melatonin of breast cancer cell proliferation with vitamin D3 is linked to activation of SMADs [35]. Melatonin also activates an osteogenic process by upregulating BMP-2 and -4 expression via the ERK and Wnt pathways [36]. We have also reported an inhibitory effect of melatonin on ACTH production by pituitary corticotrope cells, in which MT1 and BMP-4 actions were mutually enhanced [24]. In the ovary, melatonin was shown to suppress BMP-6-induced SMAD1/5/8 signaling in granulosa cells [37]. In adrenocortical cells, melatonin facilitated aldosterone production induced by ACTH combined with activin via the cAMP-PKA pathway [13]. Further studies are needed to draw a conclusion regarding the impact of melatonin on the regulation of systemic catecholamine synthesis.

As for the interaction of catecholamine and glucocorticoids, it is known that endogenous glucocorticoids induce catecholamine biosynthesis by stimulating catecholamine-synthesizing enzymes though the cortico-medullary portal system [3]. Increased expression of phenylalanine N-methyltrasferase (PNMT) [3] and TH [38] and augmented catecholamine release from adrenal glands [39] are involved in this mechanism. This is also related to the fetal programming of adult hypertension due to prenatal glucocorticoid exposure [40]. In a clinical setting, there have been several clinical reports of patients with pheochromocytomas undergoing a crisis following the administration of corticosteroids [41]. However, in patients with adrenal disorders other than pheochromocytomas, plasma levels of catecholamines were not increased by dexamethasone [42], suggesting that a glucocorticoid-induced increase in catecholamines might occur only in the situation of pheochromocytoma as a non-physiological phenomenon. Taken together, the results indicate that melatonin has inhibitory effects on ACTH-glucocorticoid secretion from the standpoint of systemic hormone regulation, whereas in the adrenal medulla,

melatonin enhanced the stimulatory effects of glucocorticoids on catecholamine synthesis, suggesting that melatonin is likely to play a regulatory role in the cortico-medullary portal system.

Collectively, the results showed that melatonin action reduced catecholamine production via the cAMP pathway and decreased catecholamine production in BMP-4-treated cells via the SMAD1/5/8 pathway (**Fig. 4**). In this mechanism, upregulation of *Bmp4*, *Alk2* and *Bmpr2* and downregulation of inhibitory *Smad6*/*7* were functionally linked to the enhancement of BMP-4-induced SMAD1/5/8 signaling in adrenal medullary cells. Melatonin also upregulated GR expression in PC12 cells, leading to the amplification of glucocorticoid-induced *Th* mRNA levels. From a clinical standpoint, this action of melatonin and the functional interaction of melatonin with glucocorticoids could be involved in the regulation of nocturnal blood pressure [26].

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Figure Legends:

Fig. 1. Expression of melatonin receptor in the rat adrenal medulla and effects of melatonin on catecholamine synthesis by PC12 cells. **A**) Total cellular RNAs were extracted from PC12 cells, rat whole adrenal tissues, and adrenomedullary tissues. Expression of *Mt1* was detected by RT-PCR analysis. Aliquots of PCR products were electrophoresed and visualized by ethidium bromide staining. MM indicates molecular weight marker. **B**) PC12 cells were treated with 100 nM of melatonin or dexamethasone for 24 h. The cell lysates were then subjected to SDS-PAGE/immunoblotting analysis using anti-MT1 and anti-ACTIN antibodies. **C**) PC12 cells were cultured in DMEM containing 1% FCS and 1% HS, and then the cells (3×10^5 cells/well) were treated with the indicated concentrations of melatonin. The culture medium and cell lysates were collected after 24-h culture, and dopamine levels were determined by HPLC and expressed as fold changes standardized by each control level. **D**) Total cellular RNAs were extracted after 24-h culture, and mRNA levels of *Th, Ddc, Dbh,* and *Rpl19* were analyzed by quantitative PCR. The expression

levels of target mRNA were standardized by *Rpl19* level in each sample, and then levels of mRNA of target genes were expressed as fold changes. **E**) Cells (3×10^5 cells/well) cultured in DMEM containing 1% FCS and 1% HS were treated with the indicated concentrations of melatonin and luzindole. Total cellular RNAs were extracted after 24-h culture, and mRNA levels of *Th* and *Rpl19* were analyzed by quantitative PCR. The expression levels of *Th* mRNA were standardized by *Rpl19* level in each sample, and then levels of *Th* mRNA were expressed as fold changes. **F**) Cells (3×10^5 cells/well) were cultured in DMEM containing 1% FCS and 1% HS with the indicated concentrations of melatonin in the presence of IBMX (0.1 mM) for 24 h. The culture medium and cell lysates were collected, and the cAMP levels were determined by an enzyme immunoassay after acetylation and expressed as fold changes standardized by each control level. Results are shown as means ± SEM. The results were analyzed by ANOVA (C, D and F) or unpaired *t*-test (E). Values with different superscript letters are significantly different at $P < 0.05$. *, $P < 0.05$ and **, $P < 0.01$ vs. control group.

Fig. 2. Functional interaction of melatonin activity and BMP-4 signaling in the regulation of catecholamine synthesis. **A**) PC12 cells (3×10^5 cells/well) cultured in DMEM containing 1% FCS and 1% HS were treated with the indicated concentrations of melatonin and BMP-4. Total cellular RNAs were extracted after 24-h culture, and mRNA levels of *Th* and *Rpl19* were analyzed by quantitative PCR. The expression levels of *Th* mRNA were standardized by *Rpl19* level in each sample, and then levels of *Th* mRNA were expressed as fold changes. **B**) After preculture in serum-free conditions with melatonin, cells (1×10^5 cells/well) were stimulated with BMP-4 for 1 h. The cell lysates were then subjected to SDS-PAGE/immunoblotting analysis using anti-pSMAD1/5/8 and anti-ACTIN antibodies. **C**) Cells (3×10^5 cells/well) cultured in DMEM containing 1% FCS and 1% HS were treated with the indicated concentrations of melatonin and BMP-4 for 8 h. Total cellular RNAs were extracted and mRNA levels of *Id1* and *Rpl19* were analyzed by quantitative PCR. The expression levels of *Id1* mRNA were standardized by *Rpl19* level in

each sample and then expressed as fold changes. **D**) Total cellular RNA was extracted from PC12 cells treated with BMP-4 or melatonin in DMEM containing 1% FCS and 1% HS for 24 h, and mRNA levels of *Mt1* or *Bmp4* and *Rpl19* were determined by quantitative PCR. The expression levels of target mRNA were standardized by *Rpl19* level in each sample, and then levels of mRNA of target gene were expressed as fold changes. **E**) Total cellular RNA was extracted from PC12 cells treated with the indicated concentrations of melatonin in DMEM containing 1% FCS and 1% HS for 24 h, and mRNA levels of *Alk2*, *Alk3*, *Bmpr2*, *Smad6* and *Smad7* were determined by quantitative PCR. The expression levels of target 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 ANOVA (A, C) or unpaired *t*-test (D, E). Values with different superscript letters are significantly different at $P < 0.05$. *, $P < 0.05$ and **, $P < 0.01$ vs. control group.

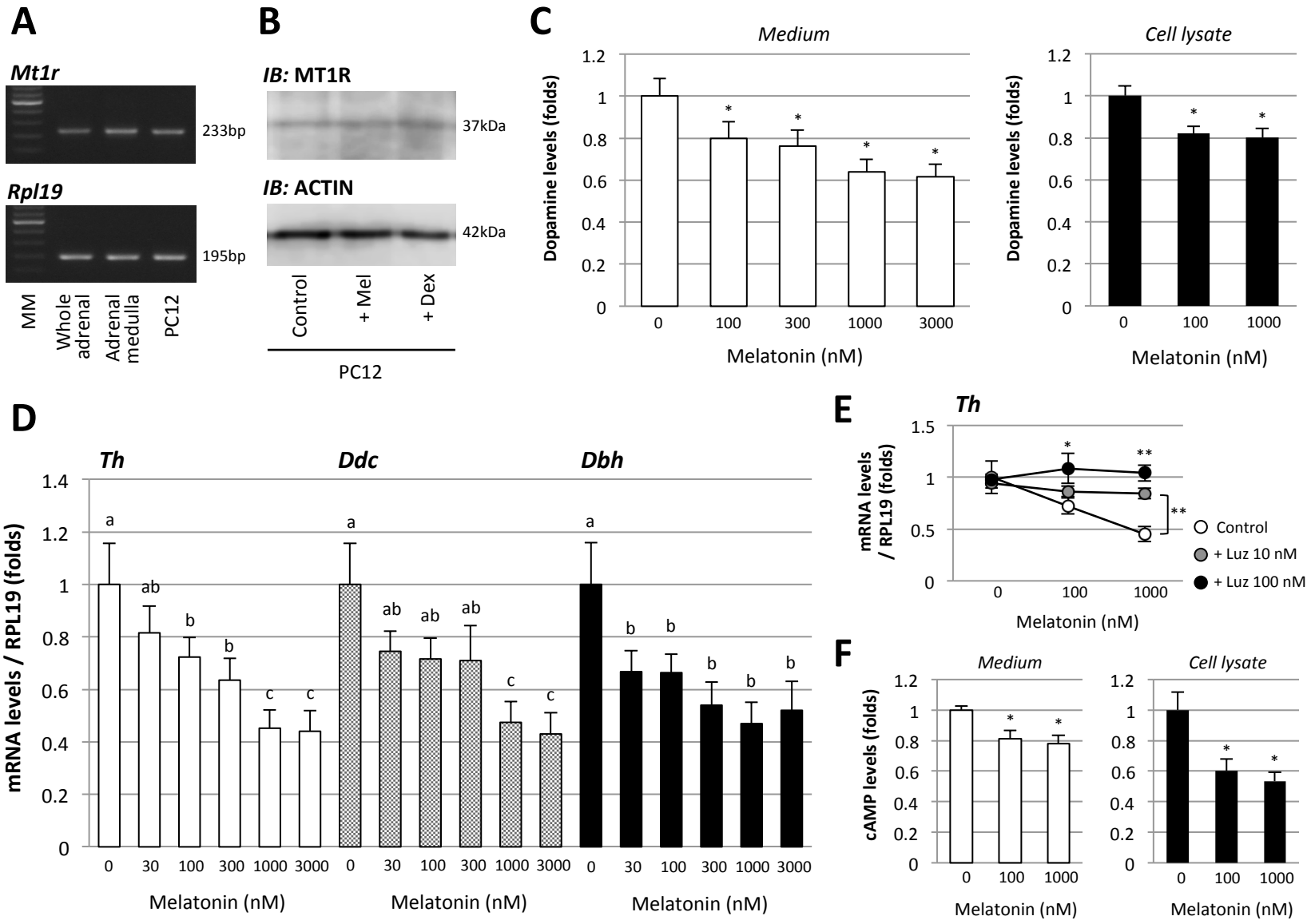
Fig. 3. Effects of melatonin on catecholamine biosynthesis induced by

adrenocortical steroids. **A, C, E**) Cells (3×10^5 cells/well) were treated with the indicated concentrations of melatonin and/or adrenal steroids including aldosterone, dexamethasone and DHT in DMEM containing 1% FCS and 1% HS for 24 h. Total cellular RNAs were extracted after 24-h culture, and mRNA levels of *Th*, *Mr*, *Gr*, *Ar*, *Bmp4* and *Rpl19* were analyzed by quantitative PCR. The expression levels of target mRNA were standardized by the level of *Rpl19* in each sample, and then levels of mRNA of target genes were expressed as fold changes. **B**) Total cellular RNAs were extracted from PC12 cells, and the expression of mRNAs encoding *Mr*, *Gr* and *Ar* was detected by RT-PCR analysis. Aliquots of PCR products were electrophoresed and visualized by ethidium bromide staining. MM indicates molecular weight marker. **D**) Cells (1×10^5 cells/well) were treated with an indicated concentration of melatonin for 24 h in serum-free conditions. The cell lysates were then subjected to SDS-PAGE/immunoblotting analysis using anti-GR and anti-ACTIN antibodies. The integrated signal density of each protein band was digitally analyzed, and the ratios of signal intensities of GR/ACTIN were calculated. Results are

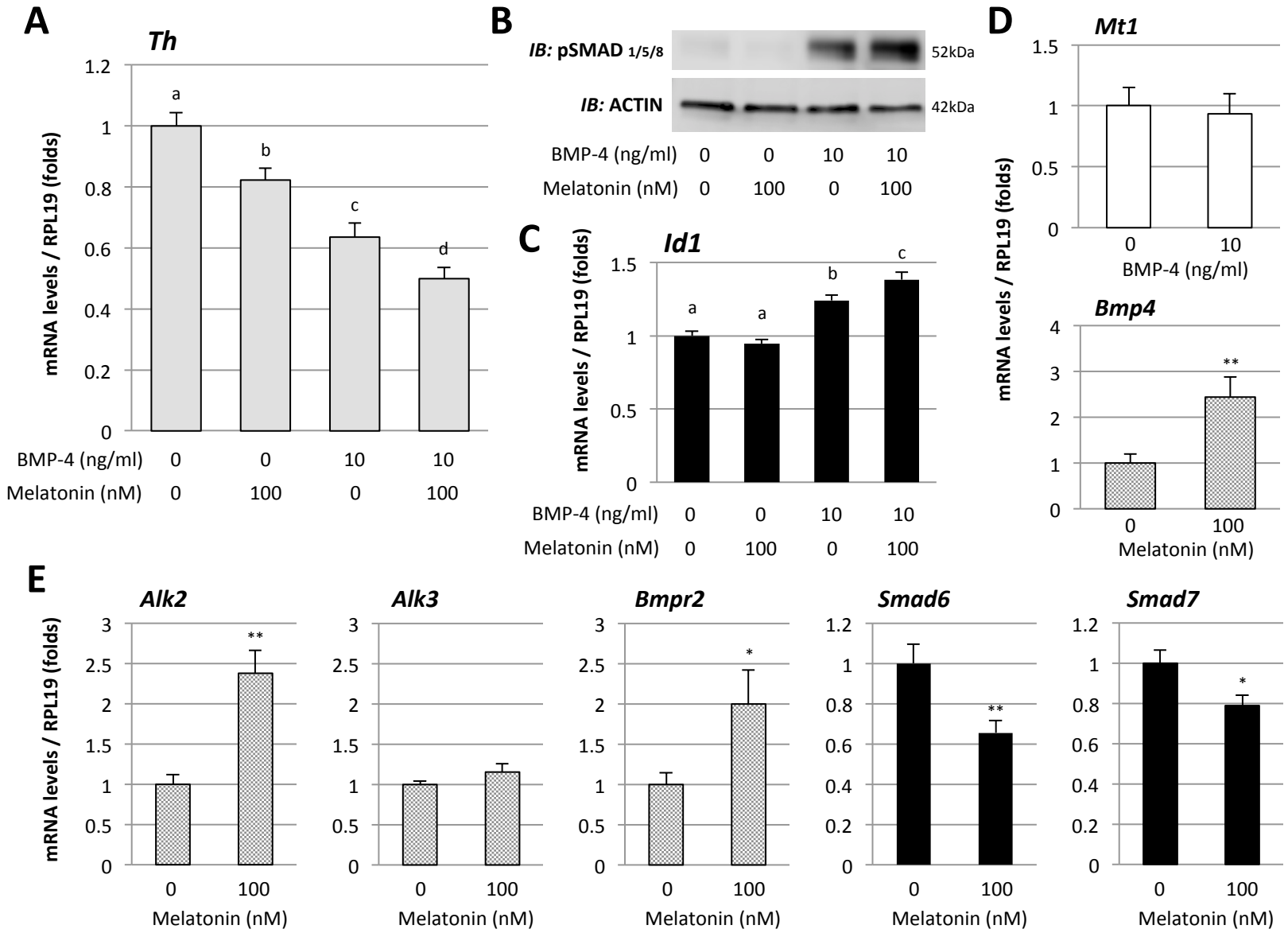
shown as means ± SEM. The results were analyzed by ANOVA (A) or unpaired *t*-test (C-E). Values with different superscript letters are significantly different at $P < 0.05$. *, $P < 0.05$ and **, $P < 0.01$ vs. control group.

Fig. 4. Possible mechanism by which melatonin and BMP-4 regulate catecholamine production under the influence of adrenocortical steroids. Melatonin suppresses catecholamine production by inhibiting the cAMP-PKA pathway in cooperation with BMP-4, in which melatonin enhances BMP-4 expression and SMAD1/5/8 signaling through upregulation of *Alk2* and *Bmpr2* and downregulation of *Smad6/7*. Among the adrenocortical steroids, melatonin preferentially facilitates the induction of catecholamine output in the presence of glucocorticoids through upregulating GR expression. Melatonin may be involved in the functional modulation of cortico-medullary interaction in the adrenal.

Rev. Fig. 1


A
Mt1r
233bp
Rpl19
195bp
MM
Whole adrenal
Adrenal medulla
PC12
B
IB: MT1R
37kDa
IB: ACTIN
42kDa
Control
+ Mel
+ Dex
PC12
C
Medium
Cell lysate
Dopamine levels (folds)
Melatonin (nM)
D
Th
Ddc
Dbh
mRNA levels / RPL19 (folds)
Melatonin (nM)
E
Th
mRNA levels / RPL19 (folds)
Control
+ Luz 10 nM
+ Luz 100 nM
Melatonin (nM)
F
Medium
Cell lysate
cAMP levels (folds)
Melatonin (nM)

Rev. Fig. 2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Th
mRNA levels / RPL19 (folds)
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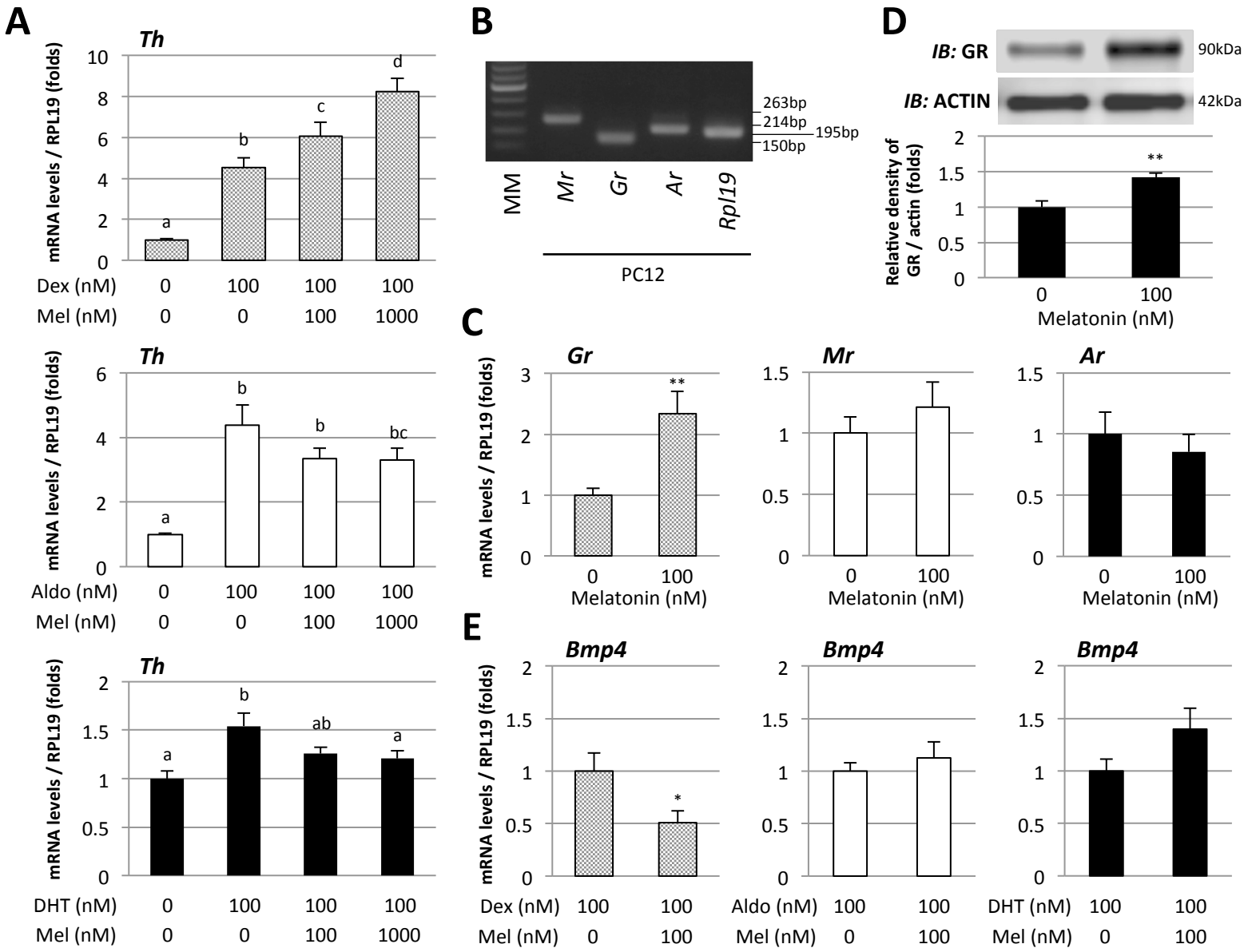
1
0.8
0.6
0.4
0.2
0
a
b
c
d
BMP-4 (ng/ml) 0 0 10 10
Melatonin (nM) 0 100 0 100
B
IB: pSMAD 1/5/8
52kDa
IB: ACTIN
42kDa
BMP-4 (ng/ml) 0 0 10 10
Melatonin (nM) 0 100 0 100
C
Id1
mRNA levels / RPL19 (folds)
1.5
1
0.5
0
a
a
b
c
BMP-4 (ng/ml) 0 0 10 10
Melatonin (nM) 0 100 0 100
D
Mt1
mRNA levels / RPL19 (folds)
1.5
1
0.5
0
0
10
BMP-4 (ng/ml)
Bmp4
4
3
2
1
0
**
0
100
Melatonin (nM)
E
Alk2
Alk3
Bmpr2
Smad6
Smad7
mRNA levels / RPL19 (folds)
**
*
**
*
0
100
Melatonin (nM)

Rev. Fig. 3


A
Th
mRNA levels / RPL19 (folds)
Dex (nM) 0 100 100 100
Mel (nM) 0 0 100 1000
Th
Aldo (nM) 0 100 100 100
Mel (nM) 0 0 100 1000
Th
DHT (nM) 0 100 100 100
Mel (nM) 0 0 100 1000
B
263bp
214bp
195bp
150bp
MM
Mr
Gr
Ar
Rpl19
PC12
D
IB: GR
90kDa
IB: ACTIN
42kDa
Relative density of GR / actin (folds)
Melatonin (nM)
C
Gr
Mr
Ar
Melatonin (nM)
E
Bmp4
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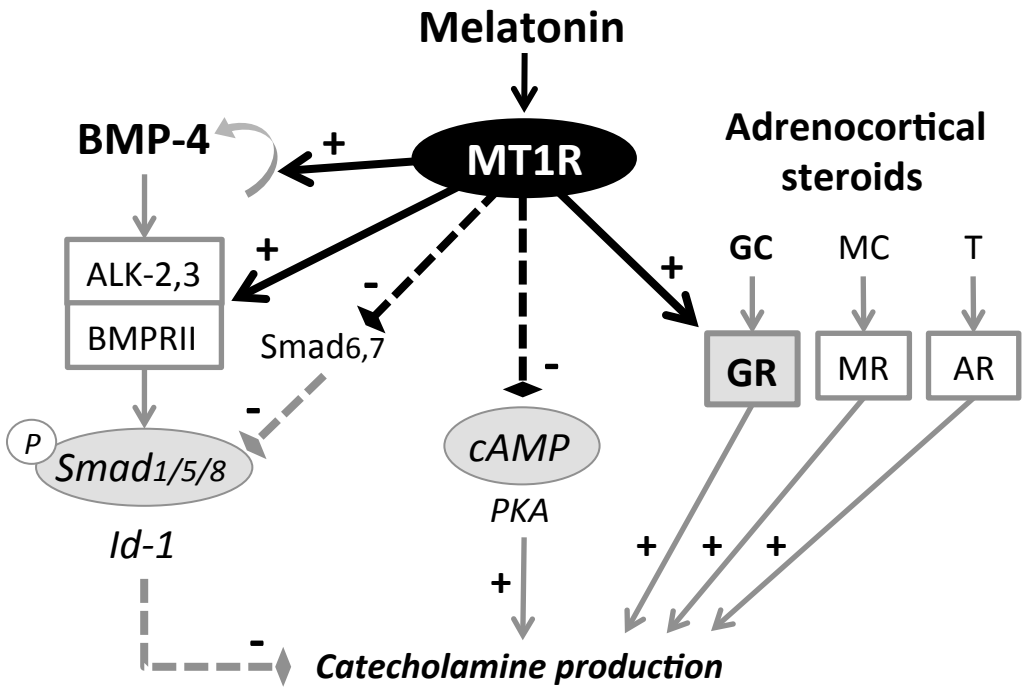
Mel (nM) 0 100
Aldo (nM) 100 100
Mel (nM) 0 100
DHT (nM) 100 100
Mel (nM) 0 100

Fig. 4


Melatonin
BMP-4
+
MT1R
Adrenocortical steroids
ALK-2,3
BMPRII
+
-
Smad6,7
+
GC
MC
T
GR
MR
AR
-
-
P
Smad1/5/8
cAMP
PKA
Id-1
+
+
+
+
-
Catecholamine production