J. Pineal Res. 2013; 54:313–321
Doi:10.1111/jpi.12019

Published by Blackwell Publishing Ltd.
Journal of Pineal Research

Journal of Pineal Research
Molecular, Biological, Physiological and Clinical Aspects of Melatonin

Analysis of the daily changes of melatonin receptors in the rat liver

Abstract: Melatonin membrane (MT1 and MT2) and nuclear (RORα) receptors have been identified in several mammalian tissues, including the liver. The mechanisms regulating hepatic melatonin receptors are yet unknown. This study investigated whether these receptors exhibit daily changes and the effects of melatonin on their levels. Our results show that mRNAs for MT1/MT2 receptors exhibit circadian rhythms that were followed by rhythms in their respective protein levels; the acrophases for the two rhythms were reached at 04:00 and 05:00 hr, respectively. Pinealectomy blunted the rhythms in both mRNAs and protein levels. In contrast, mRNA and protein levels of nuclear receptor RORα increased significantly after pinealectomy. The cycles of the latter receptor also exhibited circadian rhythms which peaked at 03:00 and 03:45 hr, respectively. Melatonin administration (10–200 mg/kg) increased in a dose-dependent manner the protein content of MT1/MT2 receptors, with no effects on RORα. Lunzindole treatment, however, did not affect melatonin receptor expression or content of either the membrane or nuclear receptors. Together with previously published findings which demonstrated the intracellular distribution of melatonin in rat liver, the current results support the conclusion that the circadian rhythms of MT1/MT2 and RORα receptors are under the control of the serum and intracellular melatonin levels. Moreover, the induction of MT1/MT2 receptors after the administration of high doses of melatonin further suggests that the therapeutic value of melatonin may not be restricted to only low doses of the indoleamine.

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Key words: circadian rhythms, melatonin, MT1, MT2, rat liver, RORα

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Received July 23, 2012;
Accepted September 21, 2012.

Introduction

Two mammalian membrane receptors, Mel 1_A and Mel 1_B (MT1 and MT2, respectively), were cloned and characterized previously [1, 2]. Both belong to the G-protein-coupled receptor (GPRC) superfamily of membrane receptors and share high homology (about 60%) [3]. Expressions of MT1 and MT2 are somewhat different: MT1 is expressed in brain (hypothalamus, cerebellum, SCN, hippocampus, substancia nigra, and ventral tegmental area) [4–8], immune system cells, testes, ovary, skin, liver, kidney, adrenal cortex, placenta, breast, retina, pancreas, and spleen [7–12], whereas MT2 is expressed in the brain (hypothalamus, suprachiasmatic nucleus), immune system cells, retina, pituitary, blood vessels, testes, kidney, gastrointestinal tract, mammary glands, adipose tissue, and the skin [2, 10, 12–14]. While the distribution of these receptors in various tissues has been well studied, the regulation of their densities has been sparingly examined.

In recent years, studies on the processes that modulate the concentration of melatonin membrane receptors have been examined in brain regions, especially in the suprachiasmatic nucleus (SCN) and pars tuberalis (PT). It was observed that the expression of these receptors varies with the species, location, light/dark cycle, time of the day, stage of neural development, and endocrine status [15]. For example, the density of melatonin receptors decreases 10-fold at 30 days of birth in the rat pars distalis (PD) [15], whereas the PT receptors are under the influence of melatonin [16]. Similar changes seem not to occur in other brain areas of the rat. Hamsters show significant daily changes in the SCN melatonin membrane receptor density, changes that are controlled by the photoperiod [17]. In contrast, the brain of other species including the salmon (*Salmo salar* L.) does not display changes in its melatonin receptors [18]. An age-dependent reduction in melatonin binding sites in the cerebral arteries [15] and decreases in 2-[125]-iodomelatonin binding and MT1 receptors in the SCN of rats and mice [19, 20] have been also reported, consistent with aging being a factor that determines melatonin receptor density.

In addition to the membrane receptors, nuclear melatonin receptors, or at least nuclear binding sites for melatonin, have been shown. These nuclear binding sites were initially pharmacologically characterized [21, 22], and soon thereafter, they were cloned and identified as belonging to the RZR/ROR orphan receptor subfamily, with includes the products of three genes: ROR_α and its splicing variants ($ROR_\alpha1$, $ROR_\alpha2$, $ROR_\alpha3$ and ROR_α) ROR_β ($ROR_\beta1$, $ROR_\beta2$) and ROR_γ ($ROR_\gamma1$, $ROR_\gamma2$) [23–26]. The ROR

subfamily exhibits a distinct spatiotemporal expression pattern, suggesting that the members of this family have different functions. ROR_α is distributed in adipose tissue, skin, testes, cartilage, and liver [27]; ROR_β has been detected in neural tissues and in the retina [25], and ROR_γ is expressed in skeletal muscle, liver, kidney, adipocytes, and the immune system [26–28]. The expression of nuclear receptors also changes with the developmental state, tissue, and endocrine status. While in the mouse, the expression of RORα receptors is maximal at 16th day of life in cerebellum, thalamus, and olfactory bulb neurons, and at 7th day in the hippocampus [29], their expression in testes was detected only after sexual maturation [30].

We recently analyzed the daily changes, subcellular distribution, and melatonin levels in the organelles of rat hepatocytes. The melatonin concentration of different subcellular organelles of liver varies with time of day, pinealectomy, and after the exogenous administration of melatonin [31]. As follow-up to this study, we considered it worthwhile to examine the daily changes in the mRNAs and protein expression of MT1, MT2, and RORα receptors in liver of rats, along with their relationship to the changes in melatonin levels under the same conditions.

Materials and methods

Animals and treatments

Three-month-old male Wistar rats were obtained from Harlan (Barcelona, Spain). They were housed under controlled temperature conditions (22 ± 1ºC) and maintained on a light/dark (LD) cycle of 12:12 hr (lights on at 07:00 hr). All experiments were performed according to the Spanish Government Guide and the European Community Guide for animal care. Animals were divided into four groups: (i) controls, (ii) sham-pinealectomized (SPx), (iii) pinealectomized (Px), and (iv) rats treated with the melatonin membrane receptor blocker, luzindole (Lz).

To study the circadian variations in the melatonin receptors, subgroups of SPx and Px rats (n = 6 animals/subgroup) were sacrificed at 08:00, 12:00, 16:00, 20:00, 24:00, 02:00, 04:00, and 06:00 hr. The sacrifice of animals at night was performed under a dim red light which does not influence endogenous melatonin production [32]. To test whether exogenous melatonin influences melatonin receptor expression or protein levels, sham-pinealectomized (SPx) and pinealectomized (Px) animals (n = 6 animals/dose) were intraperitoneally (i.p.) injected with 10, 40 or 200 mg/kg bw melatonin at 08:00 hr and sacrificed 4 hr later at noon. Some melatonin-treated animals received an i.p. injection of luzindole (4.5 mg/kg) at 07:30 hr, and the livers were collected at noon.

Melatonin was dissolved in 20% 1,2-propanediol and injected intraperitoneally, whereas luzindole was dissolved in water/ethanol 1:2 (v/v) and administered 30 min before melatonin injection. Pinealectomy was performed under equithesin anesthesia (1 mL/kg bw) following a procedure previously published [33], and the liver of these animals were studied 5 days later. Rats were sacrificed and liver was removed, washed in cold saline, and stored at −80º C until their use.

Isolation of nuclei from liver

Rat livers were washed with 1× PBS (0.01 M phosphate buffer containing 0.15 M NaCl, 0.27 mM KCl, pH 7.5), minced with scissors, homogenized in PBS/PIB 1:20 (v/v) (1× PBS/phosphatase inhibitor buffer containing PBS plus 125 M NaF, 250 mM α-glycerolphosphate, 250 mM p-nitrophenyl phosphate and 25 mM $NaVO_3$, pH 7.5) at 800 rpm with a Teflon pestle, and filtered. The homogenate was centrifuged at 300 g for 5 min at 4°C, and 0.5 mM phenylmethylsulfonylfluoride (PMSF), 0.5 mM DL-dithiothreitol (DTT), and 1 μg/mL leupeptin were added to supernatants and stored at −80°C until its use for Western blot of MT1, MT2, and RORα.

For Western blot analysis of nuclear RORα content, the pellet obtained from the above centrifugation was resuspended in 1 mL hypotonic buffer (20 mM HEPES containing 5 mM NaF, 0.5 mM $NaMoO_4$, and 0.1 mM EDTA, pH 7.5) and incubated for 15 min at 4°C. Then, 50 μL of I-GEPAL was added to the sample, vortex for 10 s, and centrifuged at 14,000 g for 1 min at 4º C. The supernatant was discarded, and the pellet was resuspended in 0.2 mL lysis buffer (20 mM HEPES containing 20% glycerol, 420 mM NaCl, 1.5 mM MgCl, 0.2 mM EDTA, 20 mM NaF, 0.5 mM DTT, 1 μg/mL leupeptin, and 0.2 mM PMSF, pH 7.9). The samples were incubated for 30 min at 4º C in constant agitation and centrifuged at 14,000 g for 10 min at 4º C. The supernatants were frozen at −80º C until their use for Western blot of RORα.

Western blot analysis

For MT1 and MT2 Western blot analysis, aliquots of the cytosol homogenates were thawed and diluted up to 25 μg of protein (45 μL), and electrophoresized on 10% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes (GE Healthcare, Barcelona, Spain), and blocked in 5% PBS-T BSA (0.01 M phosphate buffer containing 0.15 M NaCl, 0.1% v/v Tween-20, pH 7.4) for 2:30 hr. Membranes were incubated overnight with antibodies against MT1 and MT2 (1:200) (sc-13186 and sc-13177, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C in agitation, washed thrice with PBS-T for 10 min, and incubated with an anti-goat secondary antibody (sc-2020; Santa Cruz Biotechnology) during 1 hr at room temperature. For RORα, aliquots of both nuclei and cytosol homogenates were thawed and diluted up to 15 μg protein (15 μL) and electrophoresized on 7.5% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and blocked in 5% PBS-T BSA for 4 hr at room temperature. Then, the membranes were overnight incubated with the RORα antibody (1:500) (sc-28612; Santa Cruz Biotechnology) at 4º C in agitation, washed thrice with PBS-T for 10 min, and incubated with an anti-rabbit secondary antibody (1:5000) (31460; ThermoFisher Scientific, Madrid, Spain) during 1 hr at room temperature. In both cases, that is, MT1/MT2, and RORα, the membranes were washed thrice with PBS-T for 10 min, and the immunodetection was performed with a commercial chemiluminescence detection kit (34075 Super Signal West Dura; Thermo Scientific). The blots were then analyzed and

quantified with a Kodak ImageStation 2000R (Easmant Kodak Company, Rochester, NY, USA). Bradford method was used for protein measurement [34].

Reverse transcription-polymerase chain reaction

RNA was isolated from rat liver using the RNA isolation kit Real Total Spin Plus (Durviz, S.L., Valencia, Spain). RT-PCR was performed in a Stratagene 3005P system. Primer sequences (Table 1) were designed using the Beacon Designer software (Premier Biosoft Int., Palo Alto, CA, USA). RT-PCRs were carried with a final volume of 25 μL of reaction mixture containing 100, 150, and 200 ng of RNA (for MT1, MT2, and RORα, respectively), 12.5 μL of 2× MESA GREEN MasterMix Plus (Eurogentec, Seraing, Liège, Belgium), 0.4, 0.2, and 0.1 μM of specific gene primers for MT1, MT2, and RORα, respectively, and RNase-free water. PCR conditions were as follows: 5 min at 95º C followed by 40 thermal cycles at 95°C of 15 s each one, 1 min at 60º C, plus 1 min at 72º C. Data were analyzed according to the standard curves constructed with 500, 50, 5, 0.5, and 0.05 ng of RNA from MT1, MT2, and RORα and were normalized by GAPPH expression.

Statistical analysis

Data are expressed as the means ± S.E.M. One-way ANOVA with a post test was used. Cosinor analysis was performed with the Time Series Analysis-Seriel Cosinor 6.3 Lab View software (TSASC 6.3; Expert Soft Technologie Inc, BioMedical Computing and Applied Statistics Laboratory, Esvres, France). Statistical significant differences were accepted when $P < 0.05$.

Results

Fig. 1 represents the daily changes in rat liver MT1 receptors. SPx animals exhibited a circadian rhythm in both MT1 mRNA and protein levels with peaks at 04:00 hr ($P < 0.001$). Pinealectomy blunted these rhythms, and the mRNA expression and protein levels of MT1 receptor remain constant through the 24 hr. Analysis of the daily MT2 receptor changes reveals similar results (Fig. 2). Indeed, SPx rats show circadian variations in the mRNA expression and protein level of MT2, reaching the maximal values at 04:00 hr ($P < 0.001$). Pinealectomy also blunted the circadian variation in MT2 receptor. When the daily changes in RORα receptor were analyzed, the results were opposite to those of MT1 and MT2 (Fig. 3). We observed that SPx animals display minor changes in the daily mRNA expression and protein content of RORα receptor with Px increasing them.

Table 1. Sequence of the primers used for real-time PCR analysis of melatonin receptors

Genes	Sequences primers
GAPDH forward	GGC ACA GTC AAG GCT GAC AAT
GAPDH backward	ATG GTG GTG AAG ACG CCA GTA
MT1 forward	AGC CCT CTC CAC TAA TAA CC
MT1 backward	ACC CTG TAT CAC TTG TTT GC
MT2 forward	GTG TCA TTG GCT CTG TCT TC
MT2 backward	CAG TAT CCA TAT TCG CAG GTAG
RORα forward	CGA GGT ATC TCA GTC ACG AAG
RORα backward	GCC GAG GAC AGG AGT AGG

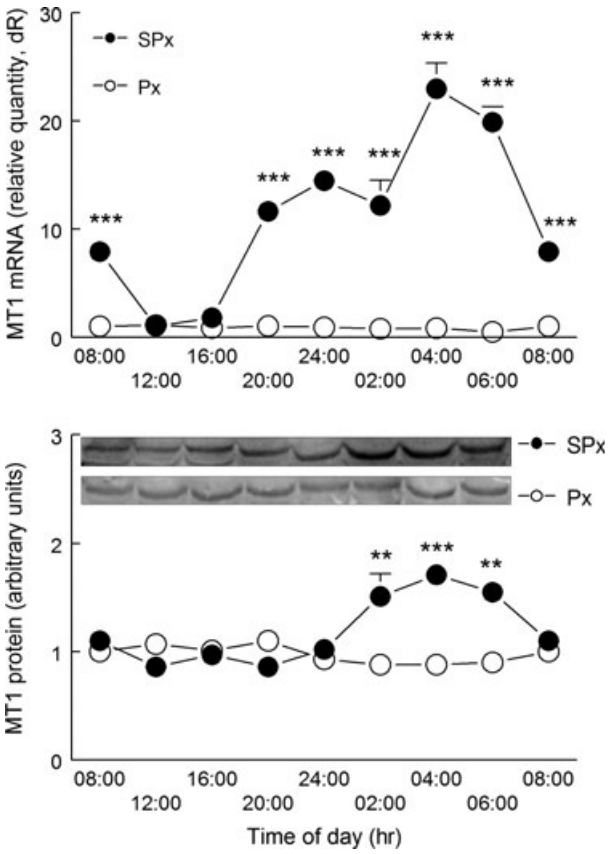

Fig. 1. Daily changes in mRNA expression (upper panel) and protein levels (lower panel) of MT1 melatonin receptor in liver of sham-pinealectomized (SPx) and pinealectomized (Px) rats. $^{**}P < 0.01$, and $^{***}P < 0.001$ versus Px.

Cosinor analysis of the MT1, MT2, and RORα rhythms in mRNAs and protein levels is shown in (Table 2). In all cases, mRNA expression precedes the maximal protein content for each receptor type.

Fig. 4 shows the mRNA expression and protein levels of MT1 and MT2 receptors after melatonin (10 mg/kg bw) and/or luzindole (4.5 mg/kg bw) administration. In all cases, the mRNA expression was measured at 12:00 hr, that is, 4 hr after that vehicle or melatonin administration. In general, it can be observed that melatonin administration enhances MT1/MT2 mRNA and protein levels, an effect augmented in the presence of luzindole. In the case of RORα (Fig. 5), the effects of Px, melatonin, and/or luzindole administration were not significant, except for the RORα protein content in the cytosol of pinealectomized animals ($P < 0.001$).

The subsequent experiments were done to assess whether high doses of melatonin modified the expression of hepatic melatonin receptors. In this case, groups of control rats were i.p. injected with 40 and 200 mg/kg bw

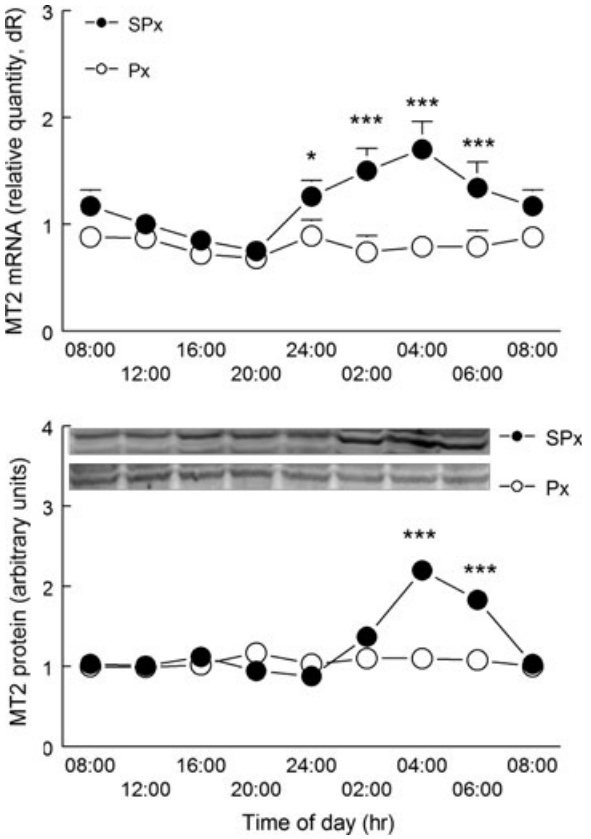

Fig. 2. Daily changes in mRNA expression (upper panel) and protein levels (lower panel) of MT2 melatonin receptor in liver of sham-pinealectomized (SPx) and pinealectomized (Px) rats. *$P < 0.05$, and *** $P < 0.001$ versus Px.

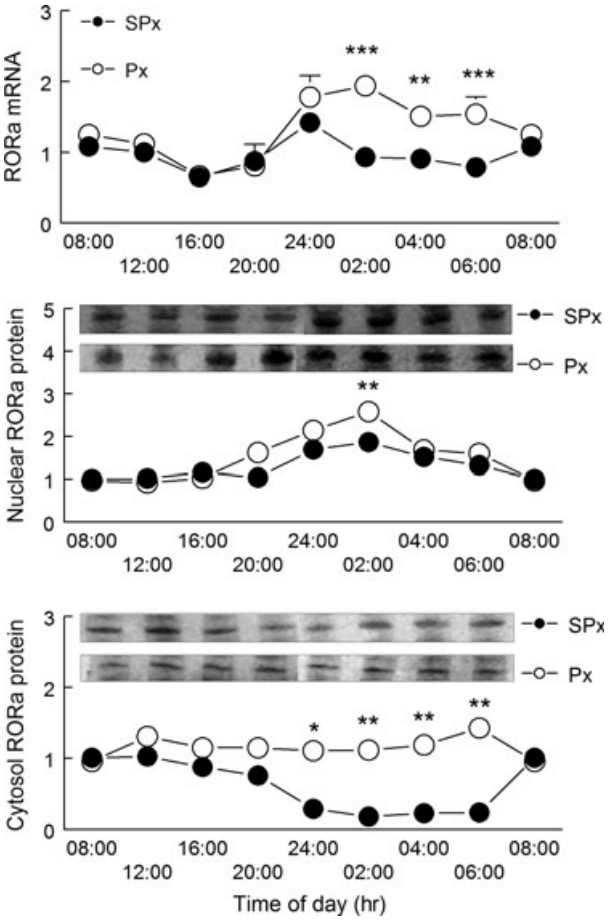

Fig. 3. Daily changes in mRNA expression (upper panel) and protein levels (lower panel) of RORα melatonin receptor in liver of sham-pinealectomized (SPx) and pinealectomized (Px) rats. See (Fig. 1) for *Y*-axis units. *$P < 0.05$, **$P < 0.01$, and *** $P < 0.001$ versus Px.

melatonin at 8:00 hr, and sacrificed 4 hr later. Fig. 6 shows that 40 mg/kg bw melatonin significantly increased MT1 mRNA expression ($P < 0.01$) and protein content ($P < 0.01$), an effect further increased with 200 mg/kg melatonin ($P < 0.001$). Melatonin administration, however, did not influence the RORα mRNA or protein levels (Fig. 7).

Discussion

The current data show, for the first time, the existence of daily variations in the mRNA and protein levels of MT1, MT2, and RORα melatonin receptors in rat liver. A cosinor analysis revealed the existence of significant rhythms for both mRNAs and protein levels. Pinealectomy blunted the rhythms of MT1 and MT2 receptors and enhanced the rhythm of RORα, whereas melatonin administration increased the MT1/MT2 protein levels in a dose-dependent manner. These data suggest that the daily changes in melatonin receptors may be under the control of blood melatonin levels derived from the pineal gland; blood levels of melatonin exhibit a nocturnal rise with highest values at 02:00 hr [31, 35].

The events leading to maximal melatonin receptor expression would be expected to be related to the circadian rhythm of melatonin, its physiologic ligand. The acrophases

Table 2. Cosinor analysis of the changes in the expression of mRNA and in protein levels of MT1, MT2, and RORα melatonin receptors in rat liver

Receptor	Acrophase	Amplitude	Mesor	Significance
MT1 mRNA				
SPx	4:01 ± 0.05	8.80 ± 0.09	9.69 ± 0.65	$P < 0.001$
MT1 protein				
SPx	5:17 ± 0.5	0.37 ± 0.07	1.12 ± 0.05	$P < 0.001$
MT2 mRNA				
SPx	4:02 ± 0.08	0.45 ± 0.1	1.11 ± 0.06	$P < 0.001$
MT2 protein				
SPx	5:10 ± 0.07	0.40 ± 0.07	1.22 ± 0.05	$P < 0.001$
RORα mRNA				
SPx	1:28 ± 0.03	0.12 ± 0.07	0.94 ± 0.05	n.s.
Px	3:32 ± 0.1	0.59 ± 0.01	1.11 ± 0.08	$P < 0.001$
RORα protein (nucleus)				
SPx	1:28 ± 0.04	0.39 ± 0.04	1.28 ± 0.03	$P < 0.01$
Px	2:16 ± 0.06	0.75 ± 0.06	1.47 ± 0.04	$P < 0.01$
RORα protein (cytosol)				
SPx	13:45 ± 0.02	0.45 ± 0.02	0.66 ± 0.03	$P < 0.05$
Px	6.14 ± 0.08	0.37 ± 0.05	1.28 ± 0.04	n.s.

Acrophase is expressed as hr:min.

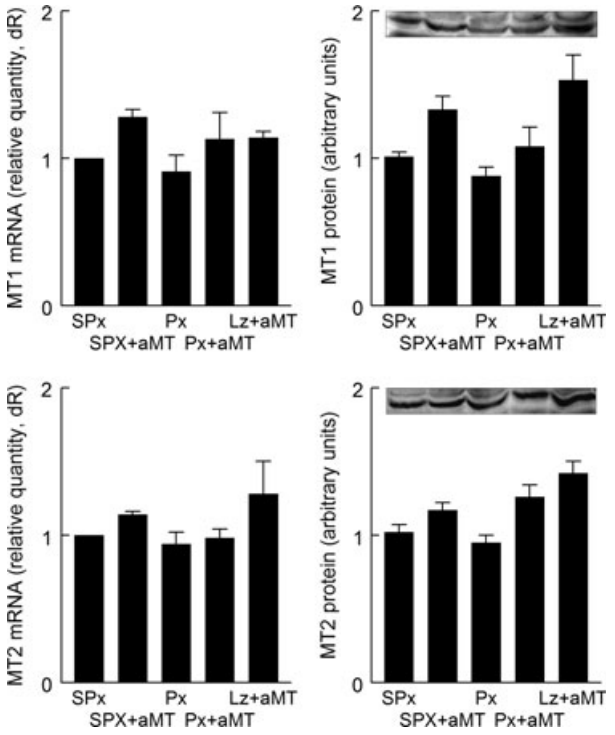

Fig. 4. Effects of sham (SPx) or pinealectomy (Px) alone or in combination with melatonin (aMT) and luzindole (Lz) plus melatonin on the MT1 and MT2 mRNA expression and protein levels in rat liver. Rats were sacrificed at 12:00 hr after being injected with vehicle (SPx and Px) or melatonin (SPx, Px and Lz) 4 hr earlier. Luzindole was given 30 min before melatonin.

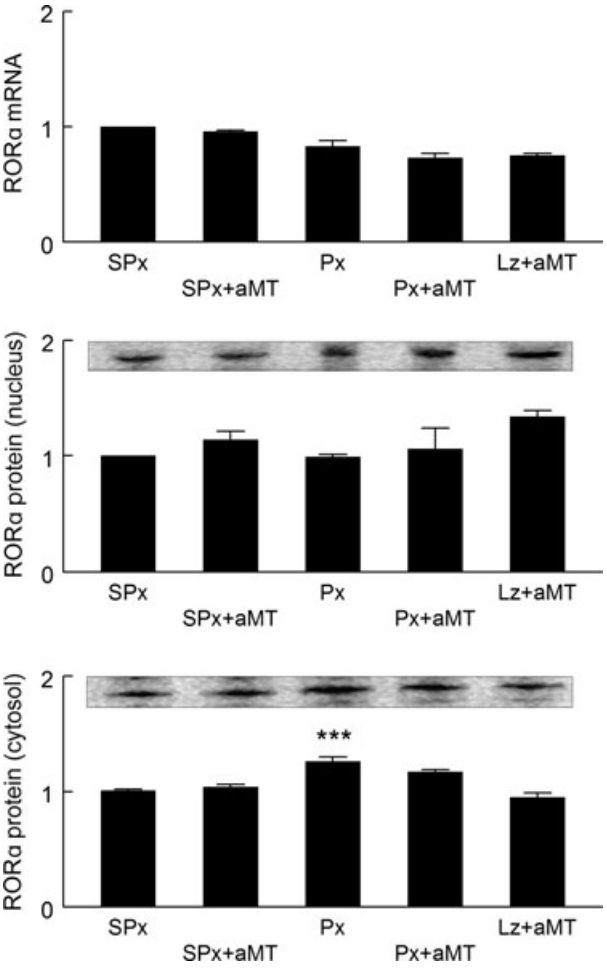

Fig. 5. Effects of sham (SPx) or pinealectomy (Px) alone or in combination with melatonin (aMT) and luzindole (Lz) plus melatonin on the RORα mRNA expression and the protein levels in rat liver cytosol and nucleus. Rats were sacrificed at 12:00 hr after being injected with vehicle (SPx and Px) or melatonin (SPx, Px, and Lz) 4 hr earlier. Luzindole was given 30 min before melatonin. See (Fig. 1) for *Y*-axis units. ***$P < 0.001$ versus SPx, Px+aMT, Lz+aMT.

of the MT1 and MT2 mRNAs expression peaked near to 03:00 hr, and 2 hr later the acrophases of the MT1/MT2 protein contents reached their maximal. In the same experimental model, we recently reported that plasma melatonin peaks at 02:00 hr, that is, 1 hr earlier than the MT1/MT2 mRNAs peak [31]. Thus, it can be suggested that the rises in the mRNAs and protein may be induced by the nocturnal plasma melatonin increase, following by MT1/MT2 mRNAs expression. At this time, that is, 03:00–05:00 hr., MT1/MT2 receptors are then activated by and respond to circulating melatonin. Soon thereafter, the reduction in circulating melatonin levels, with its ability to down-regulate its own receptors, reduce both the mRNAs expression and protein content of both MT1/MT2 receptors. If similar rhythms related to the receptors are common to many tissues, it is likely that the regulation of the melatonin rhythm by the light/dark cycle determines its ability to synchronize a variety of internal rhythms [36, 37]. This hypothesis is supported by the observation that greatly reduced plasma melatonin after pinealectomy blunted both MT1 and MT2 receptors expression.

Considering that the intracellular melatonin levels in rat liver increase after pinealectomy [31], two alternatives for melatonin–melatonin receptor interactions exist: (i) the expression of MT1/MT2 receptors is up-regulated by melatonin of pineal origin; thus, when circulating melatonin levels drop after pinealectomy, the signal to induce the mRNA expression of these receptors is lost, and (ii) increased intracellular melatonin levels after pinealectomy are responsible for the inhibition of MT1/MT2 receptors, suggesting that elevated intracellular melatonin content causes the inhibition on the mRNAs expression of these receptors. In an attempt to resolve this question, we found that after melatonin administration to rats, which increased the intracellular content of the indoleamine [31], the MT1/MT2 protein content significantly increased in a dose-dependent manner. So, whereas pinealectomy reduces plasma melatonin and increases its intracellular levels, melatonin administration increases both plasma and intracellular levels of the indoleamine. In these conditions, pinealectomy reduces and melatonin administration increases MT1/MT2 receptors. Thus, these observations are consistent with the idea that the intracellular melatonin levels are responsible for the regulation of MT1/MT2 receptor expression. In support of this assumption, it was reported that aging is followed by a reduction in both pineal and tissue melatonin levels and MT1/MT2 receptors [38–40].

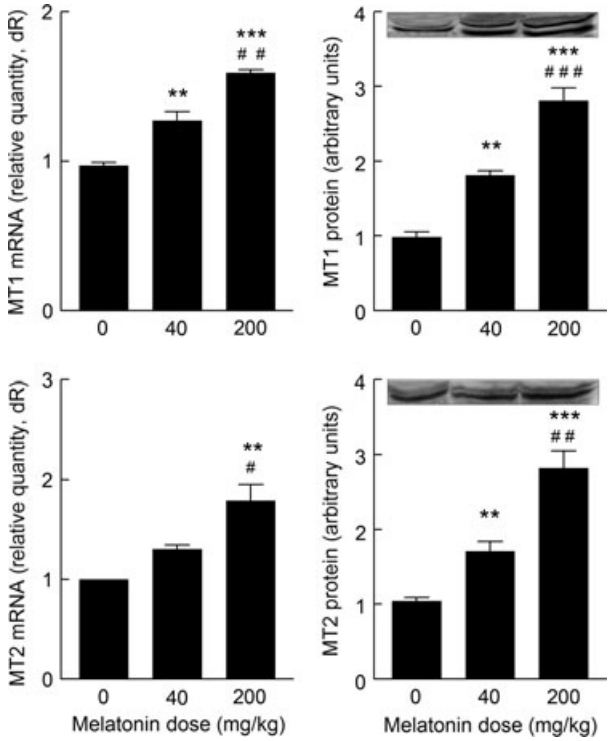

Fig. 6. Dose-dependent effects of melatonin administration on MT1 and MT2 mRNA expression and protein levels in rat liver. Rats were i.p. injected with either vehicle (0) or 40 or 200 mg/kg bw at 8:00 hr and sacrificed 4 hr later. $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus the 40 mg/kg dose.

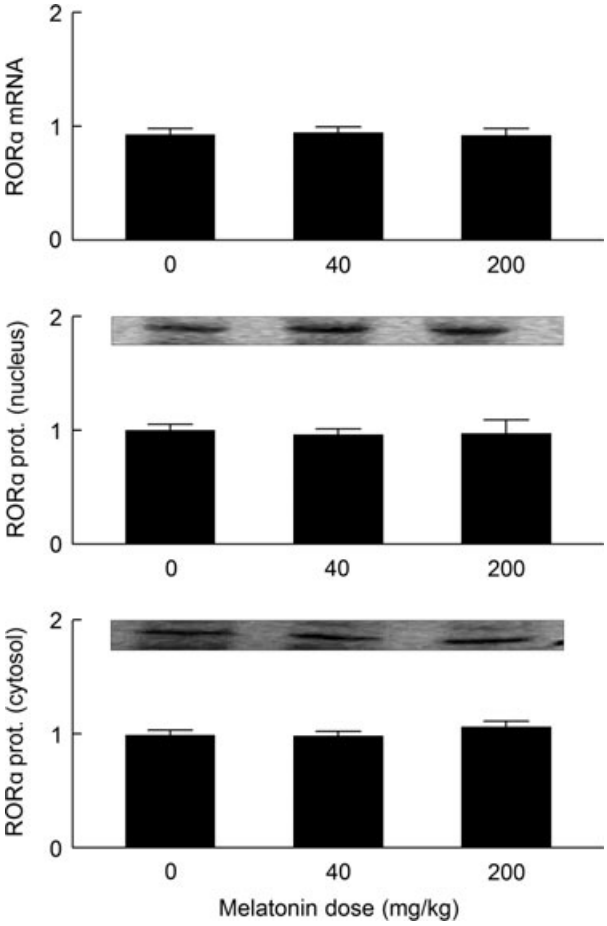

Fig. 7. Dose-dependent effects of melatonin administration on RORα mRNA expression and protein levels in rat liver. Control rats were i.p. injected with either vehicle (0) or 40 or 200 mg/kg bw at 8:00 hr and sacrificed 4 hr later. See Fig. 1 for *Y*-axis units.

The regulation of melatonin receptors may vary with the organ or tissue examined. Our results are consistent with data obtained in rat pars tuberalis (PT), which shows a nocturnal rise in MT1 receptors that is not changed by pinealectomy [16]. However, in the SCN, the regulation of MT1 receptors involves the light/dark cycle even in pinealectomized rats [41]. In other tissues including the intestine and colon, melatonin receptors do not display a diurnal rhythm, with fasting being a signal that triggers the expression of their mRNA [42]. Other examples of melatonin receptor regulation include those in the rat uterine antimesometrial stroma, where MT1 receptors are under the control of ovarian hormones [43]. In the majority of tissues, the relationship between intracellular melatonin content and MT1/MT2 mRNA expression has not been explored; the current results indicate an examination of these interactions may be informative.

Regarding the RORα nuclear melatonin receptor, we also found the presence of daily variations in the mRNA and protein levels in rat liver nucleus and cytosol, although the cosinor analysis only revealed a significant rhythm in mRNA expression in pinealectomized rats. Similar results were reported in other peripheral tissues including liver, heart, stomach, kidney, and lung of mice, where a slight oscillation in mRNA levels was detected at different photoperiods [44]. The rat SCN, however, exhibits a RORα mRNA circadian rhythm [45].These data indicate that the expression of RORα depends not only the tissue but possibly on the species studied. The decrease in RORα protein levels in cytosol together with its rise in the nucleus during the dark period in control rats, when the melatonin peak occurs, suggests that the melatonin may control the shuttle between cytosol and nucleus. This hypothesis is further supported by the data in pinealectomized rats where both the mRNA expression and nuclear protein content of RORα increased significantly with the acrophases occurring at 02:92 and 03:46 hr, respectively. The finding that the content of RORα protein in cytosol at night did not increase to the same degree as in the nucleus further supports a movement of this protein toward the nucleus. Thus, there may exist a dynamic subcellular distribution of RORα similar to that of steroid receptors [46]. A melatonin-dependent induction in RORα mRNA expression was observed in Jurkat cell nuclei with a slight movement of RORα to the cytoplasm, which may depend on melatonin itself [47]. Balancing nuclear export/import [48] is one of the mechanisms involved in the transactivation of nuclear receptors in addition to ligand binding, phosphorylation, acetylation, and ubiquitinylation [49, 50]. The current findings, however, did not show changes in the nuclear–cytosol distribution of RORα

receptors at 4 hr after melatonin administration, in agreement with previous studies which used lower doses of melatonin [45]. Thus, changes in RORα distribution in the nucleus and cytosol occurred in advance of the time we investigated the relationship and/or the expression of RORα may be dose dependent.

That mRNA of RORα increased before that of MT1/MT2 receptors indicates a potential crosstalk between nuclear and membrane receptors, although the exact relationship, if any, between these events remains to be clarified. Unexpected, however, pinealectomy enhanced the rhythm of both mRNA and protein content of RORα receptors. Considering the above discussion on the melatonin control of MT1/MT2 receptors, for the RORα, we found that pinealectomy, which reduces plasma melatonin and increases its intracellular levels, induces RORα while exogenous melatonin administration had no effect on RORα receptors. Hence, it seems that the reduction in plasma melatonin levels triggers the signal to induce RORα expression.

Administration of 10 mg/kg melatonin to SPx and Px rats did not induce significant changes in the mRNAs and protein levels of either MT1/MT2 or RORα receptors. Moreover, luzindole treatment was also without effect of these receptors. However, a significant, dose-dependent induction of the expression of mRNA and protein of MT1/MT2 receptors was seen after the administration of 40 and 200 mg/kg of melatonin, with the later dose increasing their expression by 50%. This means that when melatonin is used in high doses for therapeutic purposes, there may be increased effects of the indoleamine due to this important induction of its receptors.

A connection between membrane and nuclear receptors for melatonin seems to exist. Melatonin receptors modulate several second messengers, including cAMP, cGMP, intracellular calcium (Ca^{2+}), and phospholipids [12, 15, 51]. The activation of membrane receptors by melatonin decreases the intracellular levels of cAMP, resulting in a lowered activity of PKA that results in changes of the phosphorylation status of target proteins such as CREB [52]. Besides the cAMP-dependent cascade, melatonin receptors can couple to a stimulation of PLC-dependent signal transduction cascades and can activate PKC [53]. As CREB, RORα is a phosphoprotein and its high constitutive activity can be modulated by a change of its phosphorylation state, so regulation of RORα would be mediated through membrane receptor gene regulation by melatonin [54]. Under normal conditions, the nocturnal rise of melatonin, which is accompanied by an increase in its membrane receptors, binds to and activates them, decreasing the intracellular levels of cAMP. This results in a reduction in RORα phosphorylation and a rise of its transcriptional activity. By this means, melatonin may regulate the transcriptional activity of RORα. The reduction in the MT1/MT2 expression after pinealectomy, with the absence of circulating melatonin, increases cAMP levels, with the subsequent phosphorylation of RORα and the reduction in its transcriptional activity. After pinealectomy we found that RORα expression increases, a finding that may be compensation for the reduced melatonin. These observations agree with the fact that high levels of melatonin, as produced after its administration to rats, did not influence RORα expression.

In summary, our data show the presence of circadian rhythms in membrane and nuclear melatonin receptors, which are under the control of melatonin. The induction of the membrane receptors for melatonin after the administration of high doses of the indoleamine reinforces the fact that the therapeutic benefits of melatonin are not restricted to a low doses of this important molecule but may likely be achieved with a wide range of doses.

Acknowledgements

This study was partially supported by grants from the Instituto de Salud Carlos III (RD06/0013/0008, PI08-1664), and from the Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía (P07-CTS-03135 and CTS-101).

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