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Research Article

Expression of membrane and nuclear melatonin receptor mRNA and protein in the mouse immune system

A. Carrillo-Vico, A. García-Pergañeda, L. Naji, J. R. Calvo, M. P. Romero and J. M. Guerrero *

Department of Medical Biochemistry and Molecular Biology, The University of Seville School of Medicine, Avda. Sánchez Pizjuan 4, 41009 Seville (Spain), Fax: +34 95 490 7048, e-mail: guerrero@us.es

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Abstract. The neurohormone melatonin plays a fundamental role in neuroimmunomodulation of several mammalian species, including mice. This effect is supported by the existence of specific melatonin-binding sites in murine immunocompetent organs. Moreover, using melatonin receptor analogues, several effects of the neurohormone on mice physiology through its membrane and nuclear receptors have been described. The expression of these receptors has never been studied, despite indirect evidence showing the presence of melatonin receptor in the murine immune system. At present, the MT_1 and MT_2 membrane receptors, and nuclear receptors belonging to the RZR/ROR family have been related to the immunomodulator effect of melatonin. Here, we show the presence of membrane and nuclear melatonin-binding sites in mouse thymus and spleen, using the specific melatonin membrane (S 20098) and nuclear (CGP 52608) receptor agonist. To confirm the presence of melatonin receptors, we analyzed the presence of membrane and nuclear receptor mRNA and protein by RT-PCR, Southern blot, and Western blot. Thus, we show that MT_1 and RORα receptor mRNA and protein are expressed in both thymus and spleen, while MT_2 receptor mRNA is only detected in the thymus. This expression of melatonin receptors strongly supports the idea of an immunomodulatory role of melatonin through its receptors.

Key words. Melatonin membrane receptor; melatonin nuclear receptor; mRNA expression; protein expression; thymus; spleen; immune system.

The indolamine melatonin (N-acetyl-5-methoxytryptamine) is the main product synthesized by the pineal gland. In mammals, two major physiological functions for the indolamine have been established. Thus, melatonin plays a central role in fine-tuning circadian rhythms [1] and seasonal changes [2] through its daily nocturnal increase in the blood [3]. Additionally, melatonin shows a remarkable functional versatility exhibiting antioxidant [4], oncostatic [5], antiaging [6], and immunomodulatory [7] properties. Four mechanisms have been described for the action of melatonin: (i) interaction with membrane receptors [8], (ii) binding to nuclear receptors [9], (iii) interaction with cytoplasmic proteins [10], and (iv) antioxidant activity, including scavenging properties [11].

A functional relationship between the pineal gland and immune system in different mammalian species has been supported by many experimental findings [7, 12]. Thus, a correlation between melatonin production and circadian and seasonal variations in the immune system has been observed [13]. A circadian rhythm in the activity of lympholytic natural killer cells from spleens of rat [14] and diurnal rhythmicity of human cytokine production [15] have been described. Moreover, surgical or functional pinealectomy has been shown to promote changes in the rodent immune system [16, 17]. The effect of melatonin administration on the immune system has also been widely described. Thus, in vivo studies indicate that

* Corresponding author.

melatonin induces an increase in thymic peptide production, such as that of thymosin α_1 and thymulin in human and rats [18], as well as increasing the mitogenic response of mouse splenocytes [19]. Moreover, melatonin administration triggers Crohn's disease symptoms [20]. On the other hand, in vitro studies show that melatonin acts on immune cells by regulating cytokine production. Thus, melatonin activates T helper (Th) cells by increasing interleukin (IL)-2 production [21] as well as counteracting the inhibitory effect of prostaglandin E_2 (PGE_2) on IL-2 production in human lymphocytes [8]. Melatonin also enhances IL-12 production by monocytes driving T cell differentiation toward the Th1 phenotype and causing an increase in interferon (IFN)-γ production [22]. Finally, over the last few years, several papers have revealed the existence of extrapineal melatonin in several tissues, including the immune system. Thus, the presence of high concentrations of melatonin in human [23], mouse [23], and rat [24] bone marrow have been described.

Specific membrane and nuclear binding sites for melatonin have been described in many different immune tissues from many species of birds and mammals, including human and rodents [25–27]. Over the last few years, there has been a great advance in molecular studies on melatonin receptors. Thus, to date, three mammalian melatonin membrane receptors have either been cloned, MT_1 [28] and MT_2 [29], or affinity-purified, MT_3 [30]. Melatonin nuclear receptors belong to the RZR/ROR orphan receptor subfamily. A great amount of data suggests that receptors of the ROR/RZR group play a significant role both in processes of differentiation and in the functioning of differentiated cells and tissues [31].

Despite the presence of melatonin receptors in a wide variety of tissues, and the effects of indolamine in a great variety of physiological processes in mice, expression of the membrane and nuclear receptor in the murine immune system had not been studied. Thus, in this paper, after describing the presence of melatonin membrane and nuclear binding sites, we show, for the first time, the expression of the melatonin membrane and nuclear receptors in mouse thymus and spleen. We demonstrate that MT_1 and RORα receptor mRNA and protein are expressed in both thymus and spleen, while MT_2 receptor mRNA only is detected in the thymus.

Materials and methods

Materials

2-[^{125}I]iodomelatonin was purchased from the Radiochemical Centre (Amersham, UK). The specific activity of the radioligand was 1900–2175 Ci/mmol and was used from 60 days. Purity of the radioligand was checked by silica gel column chromatography (SGCC) and was >95%. Melatonin was purchased from Sigma (St. Louis, Mo.). CGP 52608 [1-(3-allyl-4-oxothiazolidine-2-ylidene)-4-methyl-thiosemicarbazone] and S 20098 [Servier; N-acetyl-2-(7-methoxynaphthalin-1-yl) ethylamine] were synthesized by Dr. M. Missbach (Chemical Research, Novartis Pharma, Basel, Switzerland) and were kindly provided by Dr. I. Wiesenberg (Pharma Research, Novartis Pharma, Basel, Switzerland). All reagents used in RT-PCR, Southern blot, and immunodetection studies were purchased from Promega (Madison, Wis.). The primers and probes used in the work were purchased from Roche (Mannheim, Germany).

Experimental animals

All mouse studies (male Swiss mice between 6–8 weeks old) were conducted in accordance with the principles and procedures outlined by 'Ethical principles and guidelines for scientific experiments on animals' of the Swiss Academy of Medical Sciences.

Cell and tissue preparations

After animals had been killed by decapitation between 1200 and 1400 h, the organs were carefully removed and total RNA isolations were performed immediately. Cell suspensions from mouse thymus and spleen were prepared by mechanical dissociation. The isolated cells were washed with 0.15 M NaCl, finally resuspended in 0.15 M Dulbecco's phosphate-buffered saline (PBS), 1 mM $CaCl_2$, pH 7.4, and used immediately for binding experiments. Viability, as determined by trypan blue exclusion, was always greater than 95%.

Binding studies

In standard conditions, cells from mouse thymus and spleen (1×10^6 cells/ml) were incubated with [^{125}I]melatonin (100 pM) in incubation buffer and, when necessary, native melatonin or other compounds in a total reaction volume of 0.4 ml. The reaction was initiated by the addition of 0.2-ml aliquots of cells and performed at the times and temperatures indicated. Cell-bound [^{125}I]melatonin was separated by centrifugation, then washed with incubation buffer containing 10% (w/v) sucrose, and the radioactivity associated with the cells was measured in an LKB gamma counter. No detectable degradation of [^{125}I]melatonin during the incubation occurred, as SGCC of aliquots of the incubation medium, taken before and after incubating, revealed similar amounts of radioactivity (95%) in the position of [^{125}I]melatonin.

RT-PCR of MT_1, MT_2 and RORα receptor

mRNA was isolated by the guanidium method [32]. Single-stranded cDNA was then synthesized from cells using the following method. Ten micrograms of RNA was preincubated with 1 µg of oligo(T)15 in 20 µl RNase-free H_2O at 85 °C for 10 min and then rapidly chilled on ice. Then, 1 µl RNasin (40 U/µl), 8 µl 5× RT buffer, 8 µl

dithiothreitol (100 mM) and 2 µl deoxyribonucleotides (dNTP; 10 mM of each) were added and the mixture was incubated at 42 °C for 3 min. Finally, 1 µl Moloney murine leukemia virus reverse transcriptase (Mo-MuL-VRT; 200 U/µl) was added to give a final volume of 40 µl and the reaction was incubated at 42 °C for 60 min, then terminated by placing it on ice after deactivation at 95 °C for 5 min. RNA used for RT-PCR analysis of MT_2 melatonin receptor mRNA expression was treated with DNAase (RQ1 RNase-free DNase, 1 U/µg RNA) to test DNA contamination before cDNA synthesis. The following oligonucleotides (5′ to 3′) were used as primers for MT_1 PCR: CCG CAA CAA GAA GCT CAG GAA CTC (exon 1) and TCG TAC TTG AGG CTG TGG CAA ATG (exon 3); they amplified a single 248-bp band. For MT_2 PCR the following oligonucleotides were used: TAC ATC AGC CTC GTC TGG CTC C (forward) and TTC CTC GTA GCC TTG GCC TTC C (reverse); they amplified a single 239-bp band. And for RORα PCR the followed primers were used: GGA AGA GCT CCA GCA GAT AAC G (exon 4) and GCT GAC ATC AGT ACG AAT GCA G (exon 7), which amplified a single 417-bp band. For PCR reactions (1× PCR reaction buffer, 1.5 mM $MgCl_2$, 400 µM dNTPs), 5 µl of RT product were amplified after a 'hot start' procedure in a final volume of 25 µl using 2.5 U Taq-DNA-polymerase. Thirty PCR cycles were performed (94 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min), followed by a final 5 min extension at 72 °C. Specific primers for human hprt were used to test the efficiency of reverse transcription. The hprt primers were (5′ to 3′); GTA ATG ATC AGT CAA CGG GGG GAC and CCA GCA AGC TTG CAA CCT TAA CCA (174-bp fragment).

Southern blot

After amplification, 5 µl PCR reaction was electrophoresed in an agarose gel. The cDNA was transferred to a hy+ nylon membrane (Amersham-Pharmacia Biotech, Uppsala, Sweden). Blots were prehybridized at 68 °C for 1 h in prehybridization buffer (5× SSC, 0.1% N-lauryl-sarcosyl, 0.02% SDS, 1% blocking reagent). The hybridization was performed at 60 °C overnight in the same prehybridization buffer containing 25 ng/ml of labeled probe with an oligonucleotide tailing kit (Roche). Thereafter, blots were washed, blocked and incubated for 30 min in anti-DIG-AP (anti-digoxigenin conjugated to alkaline phosphatase). Finally, they were washed and incubated in CSPD. Blots were then exposed to Kodak X-OMAT AR film at room temperature. The probes used in this study were: MT_1 probe: TGC CAC AGC TAA ACT CAC CAC AAA TAT ATT; MT_2 probe: ACA AAG AAA TTG GGC ACC AAA GCC ACC AGA GT, and ROR_α probe: CTG AGA GTC AAA GGC ACG GCA CAT CCT AAT. All of them were directed against their own fragment amplified by PCR.

Immunodetection of MT_1 and RORα proteins

Tissues were washed with ice-cold PBS and then homogenized with a Polytron homogenizer (Kinematica, Luzern, Switzerland) at 4 °C in HEPES 20 mM, pH 7.4, containing protease inhibitors. The tissue homogenates were then centrifuged for 10 min at 4000 g and supernatants were collected to assay protein expression. The protein content of the supernatants was determined by the Bradford method [33]. Samples were run into the SDS sample buffer on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Blots were blocked and then incubated using a 1:500 dilution in block buffer of purified antisera against MT_1 for 2 h at room temperature (RT). After a Tris-buffered saline Tween-20 (TBST) washing procedure, the blots were incubated with 1:2000 peroxidase-labeled anti-rabbit antibody (Amersham-Pharmacia Biotech) in TBST for 1 h at RT. The immunodetection was performed using the enhanced chemiluminescence ECL system (Amersham-Pharmacia Biotech). For immunodetection of RORα, samples were run into the SDS sample buffer on an 8% SDS-polyacrylamide gel. Western analysis was carried out using a 1:500 dilution in block buffer of specific polyclonal antibody against RORα1 (RORα1, sc-6062; Santa Cruz Biotechnology, Santa Cruz, Calif.) for 4 h at RT. After a TBST washing procedure, the blots were incubated with 1:1000 peroxidase-labeled anti-goat antibody (DAKO, Glostrup, Denmark) in TBST for 1 h at RT. Immunodetection was performed using the ECL system.

Results

Specific melatonin membrane and nuclear binding sites

To prove the existence of specific binding sites for melatonin in mouse thymus and spleen, we studied whether different analogues of the melatonin receptor displaced the binding of 2-[^{125}I]iodomelatonin to thymocytes and splenocytes. The binding was displaced significantly by analogues of the melatonin membrane (S 20098) and nuclear (CGP 52608) receptors (fig. 1).

Gene expression of MT_1, MT_2 and RORα melatonin receptors

As we know that mouse thymocytes and splenocytes possess both membrane and nuclear binding sites (fig. 1), we asked if these cells were able to express melatonin membrane and nuclear receptors. To determine which of the two better-known mouse melatonin membrane receptor subtypes may be expressed in these cells, PCR primers specific for both melatonin subtypes (MT_1 and MT_2) were used in an RT-PCR reaction of RNA isolated from mouse thymus and spleen. Primers used for the amplification of the MT_1 receptor were located in different

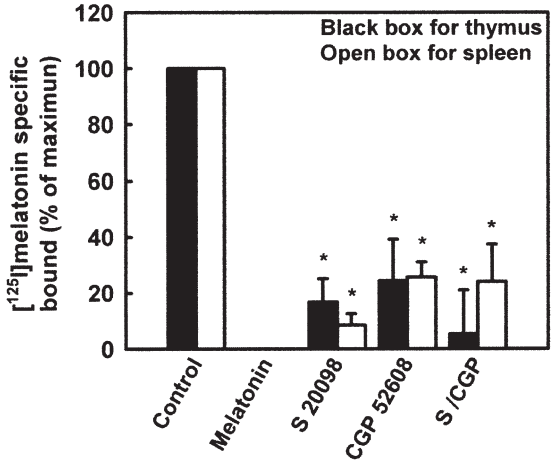

Figure 1. Binding of [^{125}I]melatonin in the presence of different analogues in mouse thymus (black bars) and spleen (white bars). Cells (1×10^6 cells/ml) were incubated with 100 pM [^{125}I]melatonin in the presence of 0.1 mM of each analogue at 15 ºC for 45 min. Bound (%) is the percentage of [^{125}I]melatonin added to the incubation medium bound specifically to the cells. Results are the mean of three experiments performed in triplicate. Statistical significance was determined by one-way analysis of variance (ANOVA test) followed by the Student-Newman-Keuls multiple-range test. * $p < 0.001$ vs control.

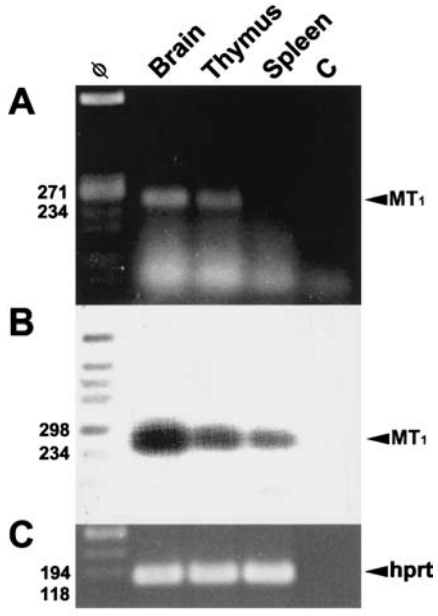

Figure 2. Analysis of MT_1 melatonin receptor mRNA expression in thymus and spleen. Brain was used as a positive control. ϕ shows molecular-weight markers (ϕX174/*Hae*III for PCR and VI DIG-labeled for Southern blot), and C shows the PCR reaction without cDNA substrate. Representative figure from ten replicates. (*A*) RT-PCR analysis of the MT_1 receptor. (*B*) Southern blot hybridization of the PCR products shown in *A* with the DIG-labeled MT_1 melatonin receptor-specific probe. (*C*) RT-PCR analysis of hprt mRNA as a housekeeping gene.

exons, while primers used for the amplification of the MT_2 receptor belonged to a known partial fragment, thus, RNA used for RT-PCR analysis of MT_2 melatonin receptor mRNA expression was treated with DNAase to test DNA contamination before cDNA synthesis. Agarose gel electrophoresis demonstrates PCR products of the expected size in thymus amplified with the MT_1 primers (248 bp), but not in spleen (fig. 2A). When we used MT_2 primers, no band was detected in either tissues (fig. 3A). To determine the presence of nuclear receptors in both tissues, PCR primers specific for RORα nuclear receptor were used in an RT-PCR reaction of RNA isolated from mouse thymus and spleen. Primers used for the amplification were located in different exons. A specific band of the expected size (417 bp) was observed in both thymus and spleen (fig. 4A). RT-PCR reactions were processed with hprt housekeeping gene primers (figs 2C, 3C, and 4C). Afterwards, we performed a Southern blot analysis employing specific DIG-labeled PCR-amplified DNA fragment MT_1, MT_2, and RORα probes. Because of the high sensitivity of Southern blot compared to PCR, we were able to confirm the identity of PCR-amplified DNA fragments and observed a hybridization signal for MT_1 receptor in spleen (fig. 2B) and for MT_2 (239 bp) in thymus (fig. 3B) while no hybridization signal of MT_2 was observed in spleen (fig. 3B). In all experiments, mouse brain was used as positive control. Nucleotide sequences of the melatonin receptor fragments amplified from the brain, thymus, and spleen were studied by direct sequencing (Newbiotechnic, Seville, Spain). Results showed that both thymus and

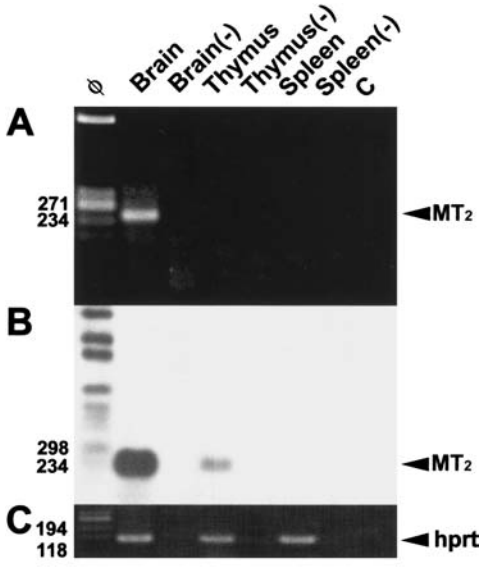

Figure 3. Analysis of MT_2 melatonin receptor mRNA expression in thymus and spleen. Brain was used as a positive control. Brain (–), thymus (–), and spleen (–) were processed in the absence of MMLV retrotranscriptase. ϕ shows molecular-weight markers (ϕX174/*Hae*III for PCR and VI DIG-labeled for Southern blot), and C shows the PCR reaction without cDNA substrate. Representative figure from ten replicates. (*A*) RT-PCR analysis of the MT_2 receptor. (*B*) Southern blot hybridization of the PCR products shown in *A* with the DIG-labeled MT2 melatonin receptor-specific probe. (*C*) RT-PCR analysis of hprt mRNA as a housekeeping gene.

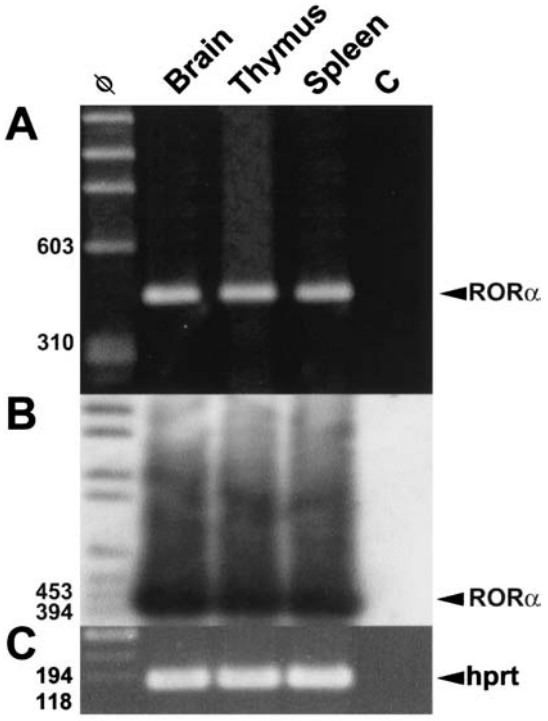

Figure 4. Analysis of RORα melatonin receptor mRNA expression in thymus and spleen. The brain was used as a positive control. ϕ shows molecular-weight markers (ϕX174/*Hae*III for PCR and VI DIG-labeled for Southern blot), and C shows the PCR reaction without cDNA substrate. Representative figure from ten replicates. (*A*) RT-PCR analysis of the RORα receptor. (*B*) Southern blot hybridization of the PCR products shown in *A* with the DIG-labeled RORα melatonin receptor-specific probe. (*C*) RT-PCR analysis of hprt mRNA as a housekeeping gene.

spleen melatonin receptor cDNA fragments exhibited identical nucleotide sequences with the mouse brain receptors. Taken together, these data show the presence of membrane and nuclear receptor in the murine immune system. Thus, we detected MT_1 mRNA expression in thymus and spleen, while we only detected MT_2 mRNA expression in thymus. Both thymus and spleen express the nuclear receptor RORα.

Protein expression of MT_1 and RORα1 receptors

Due to the presence of MT_1 (fig. 2), MT_2 (fig. 3), and RORα (fig. 4) mRNA in thymus and spleen, we wanted to determine whether this mRNA expression correlated with protein expression of these receptors. Thus, we studied protein expression of melatonin receptors expressed in the membrane and in the nucleus by Western blot analysis. We used specific rabbit antisera against the MT_1 melatonin receptor to determine MT_1 protein expression in thymus and spleen. The Western blot analysis was positive: acrylamide gel electrophoresis showed a band of the expected weight, about 37 kDa (fig. 5A). RORα1 protein was also observed in thymus and spleen (fig. 5B). We detected a protein with a weight between 50 and 55 kDa,

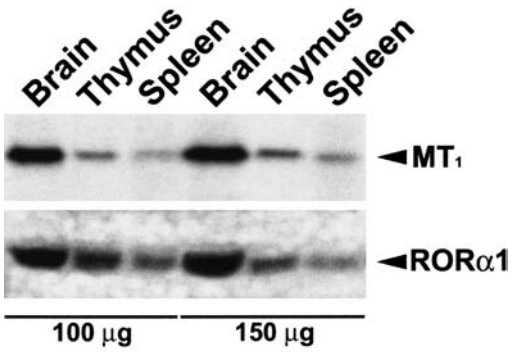

Figure 5. Western blot analysis of MT_1 membrane and RORα1 nuclear receptor. Indicated concentrations of protein from mouse thymus and spleen were separated by 12% (MT_1) or 8% (RORα1) SDS-PAGE, and then transferred to nitrocellulose and immunoblotted with specific purified rabbit antisera. Brain was used as a positive control. Representative figure from ten replicates.

which agrees with previous studies [34]. In these Western blot analyses, mouse brain was also used as a positive control.

Discussion

A large amount of evidence supports a role for melatonin in the murine immune system. Thus, in vivo administration of melatonin has been observed to enhance antibody-dependent cellular cytotoxicity [35], to enhance the antibody response to a T-dependent antigen in young or immunodepressed animals through an increase in Th cell activity and IL-2 production [36], and to reverse lipopolysaccharide-induced hyperalgesia [37]. Moreover, melatonin has been shown to inhibit apoptosis during early B cell development in bone marrow [38] and to stimulate the gene expression of immunomodulating cytokines [39]. Melatonin has also been reported to promote rejuvenation of degenerative thymus [40, 41]. In addition, in vitro studies have revealed that melatonin increases IFN-γ production by murine splenocytes and increases the ability of spleen cells to proliferate in response to mitogens [42].

An essential fact that supports a relationship between melatonin and the immune system is the presence of melatonin receptors in immune organs and cells. Although specific binding sites for melatonin had been characterized in murine thymus [43] and spleen [44], the subcellular localization of these binding sites had not been studied in any depth. In the present work, we show that both thymocytes and splenocytes present melatonin-binding sites in plasma membrane and nuclei (fig. 1).

Currently, two high-affinity membrane receptors for melatonin have been cloned in mice. These receptors, MT_1 [45] and MT_2, [46] are members of the G protein-coupled receptor superfamily, while, a third membrane

receptor, MT_3, which belongs to the family of quinone reductases, has been recently identified in mouse spleen [47]. Melatonin nuclear receptors, belong to the RZR/ROR orphan receptor subfamily, which includes the products of three genes; splicing variants of RORα (RORα1, RORα2, RORα3, RZRα), RZRβ, and RORγ. Only RORα [48] and RORγ [49] receptors have been specifically identified in mice.

Much evidence reveals that melatonin, acting through its receptors, is involved in numerous physiological processes in rodents [50–52]. As an immunomodulator, melatonin has been reported to enhance cell-mediated and humoral immunity via its MT_2 receptor [52]. Moreover, administration of luzindole, a high-affinity melatonin membrane receptor antagonist, has been shown to attenuate the ability of melatonin to enhance splenic lymphocyte proliferation [53] and suppress experimental autoimmune encephalomyelitis [54]. Antiproliferative effects of melatonin have also been shown in LNCaP human prostate and murine colonic cancer through its MT_1 and RZR/ROR receptors, respectively [55, 56].

Because of the existence of membrane and nuclear melatonin-binding sites shown in figure 1, together with the numerous biological actions exerted by melatonin on the mouse immune system and the presence of melatonin synthesis in the murine immune system, we thought an expression study of membrane and nuclear melatonin receptors in the murine immune system was necessary. We studied the expression of MT_1 and MT_2 membrane receptors and ROR_α nuclear receptor in thymus and spleen of mice, since melatonin has been postulated to exert its immunomodulatory actions through these receptors. Studies revealed the presence of MT_1 and ROR_α receptor mRNA in both thymus and spleen (figs. 2, 4), while MT_2 mRNA was only detected in thymus (fig. 3). Protein expression studies indicated clear protein expression of both membrane and nuclear receptors in thymus and spleen (fig. 5). These data show, for the first time, the presence of membrane and nuclear melatonin receptor mRNA and protein in the mouse immune system. In conclusion, the data shown in this paper, together with the development of receptor-specific analogues and the use of melatonin receptor knockout animals will contribute to understanding and elucidating the mechanisms of melatonin through its target sites in the immune system.

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