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Melatonin synthesis and melatonin-membrane receptor (MT1) expression during rat thymus development: role of the pineal gland

Abstract: To gain insight into the relationship between thymus and pineal gland during rat development, the melatonin content as well as the activity and expression of the two key enzymes for melatonin biosynthesis, i.e. N-acetyltransferase (NAT) and hydroxyindole-O-methyltransferase (HIOMT), were studied in the thymus at fetal and postnatal stages. Moreover, melatonin-membrane receptor (MT1) expression was also analyzed. We found both the expression and activity of thymic NAT and HIOMT at 18 days of fetal life. Additionally, there is production of melatonin in the thymus as well as MT1 expression at this fetal age. These results show values higher in day-time than at night-time. The pineal gland begins to produce significant levels of melatonin around postnatal day 16, and this synthesis shows a circadian rhythm with high values during the dark period; therefore the nocturnal serum melatonin may inhibit thymic melatonin production. To document this, we report an increased melatonin content of the thymus in pinealectomized rats compared with sham-pinealectomized. In conclusion, these results show, for the first time, the presence of the biosynthetic machinery of melatonin and melatonin production in developing rat thymus and that the pineal gland may regulate this process.

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Introduction

Melatonin was first isolated from the pineal gland [1] and was synthesized from serotonin by the sequential action of N-acetyltransferase (NAT) and hydroxyindole-O-methyltransferase (HIOMT) [2]. The synthesis and release of melatonin display a circadian rhythm with greatly elevated serum levels during the night in response to signals from an endogenous oscillator that is entrained by photoperiodic stimuli. The switch between day and night profiles of this neurhormone is driven by changes in the expression, protein, and activity of NAT, which increases at night 10- to 100-fold [3]. In contrast, the nocturnal increase in pineal HIOMT activity is weak but significant. HIOMT gene expression is high during daytime but displays an additional twofold increase at night [4]. In reference to the ontogeny of melatonin synthetic pathway in the rat pineal gland, it is well known that pineal melatonin production is not evident until postnatal day 5, after HIOMT and NAT activities are present [5, 6].

In addition to the pineal gland, there is increasing evidence for extrapineal melatonin synthesis in a number of tissues, including retina, Harderian gland, gastrointestinal tract, reproductive organs, skin, platelets and several brain regions [7]. Regarding the immune system, melatonin production was demonstrated in lymphoid organs and human cultured lymphocytes [8–11].

It now seems clear that the thymus and the neuroendocrine system influence the maturation of each other during early ontogeny and perinatal life in birds and mammals, including humans. The pineal gland, via the rhythmic synthesis and release of melatonin, influences the development, differentiation and function of the immune system. In vivo and in vitro data confirming this relationship have been provided as follows: (i) a correlation between melatonin production and circadian and seasonal variations in the immune system; (ii) effects of surgical or functional pinealectomy on the immune system; (iii) effects of melatonin administration in vivo on the immune system; (iv) in vitro regulation of immune cell activity by melatonin, and (v) presence of melatonin receptors in the immune system [12].

This immunoregulatory action of melatonin appears to exert these effects through specific receptors. Membrane-bound melatonin-binding sites have been identified. Thus, MT_1, MT_2, and MT_3 (ML1) or high-affinity receptors, which are coupled to GTP-binding proteins and ML2 or low-affinity receptors, are found in the lymphatic system [13]. We have previously described the presence of high-affinity binding sites for melatonin in human lymphocytes [14, 15], and in rat thymocytes and splenocytes [16]. We have also reported that melatonin interacts with purified cell nuclei from rat spleen and thymus [17], and there is a high expression of RZRα mRNA in the human and rodent

immune system [18, 19]. Moreover, we have shown that melatonin is able to enhance production of IL-2, IL-6 and IL-12 via membrane and nuclear receptors [20–23] and also increases the production of thymic peptides such as thymosin α_2 and thymulin [24].

Thus far, little is known about the developmental aspects of melatonin synthesis in extrapineal tissues, including the immune system. We thought that ontogenetic studies would be of special interest in order to delineate at which stage of development the thymus is able to synthesize melatonin. Therefore, we focused on melatonin synthesizing enzymes activity/expression and melatonin content during fetal and postnatal developing thymus. Moreover, we investigated a possible regulation of the pineal gland on melatonin synthesis in developing thymus and on melatonin receptor expression. Here, we demonstrate an inverse rhythm in melatonin synthesis in thymus compared with the pineal gland peaking during the light period of 24-hr light/dark cycle and a decreased synthesis around the third postnatal week when melatonin pineal production initially reaches its highest values.

Material and methods

Animals and tissues

Female and male Wistar rats (born in our animal facilities) were housed under controlled photoperiods (14 hr light: 10 hr darkness) with food and water ad libitum. Males were caged with females in the estrous phase of the estrous cycle for one night. The day after mating was counted as day 0. For fetal studies, pregnant rats were killed by decapitation between 12:00 and 16:00 hours (daytime) or 24:00–02:00 hours (nightime), and thymuses from fetal day 18 (F18) were removed. For postnatal studies, rats were killed by decapitation on days 7, 16, and 60 after birth (P7, P16, and P60, respectively) at the same times and thymuses were collected. After a brief rinse in normal saline solution, the thymus were removed and rapidly frozen on solid CO_2 and stored at −80°C until used for RNA extraction, enzyme activities, and melatonin determinations. At night, the thymus was obtained after killing animals under dim red light.

When required, animals were pinealectomized (Px). Neonatal pinealectomy was performed within 48 hr after birth using deep hypothermia as an anesthetic. Sham-pinealectomized (Sh) neonates were used as controls. The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy of Science, Bethesda, MD, USA).

RNA extraction and first-strand cDNA synthesis

Total thymus RNA at the four different developmental stages of the study was extracted by the guanidinium isothiocyanate method [25] using solution D and appropiate volumes of phenol and chloroform. After cell lysis and RNA extraction, RNA was precipitated with isopropanol, and the pellet was washed in 85% ethanol. The RNA samples were recovered by centrifugation at 12,000 g for 10 min and then dried. Each RNA pellet was raised in 400 μL RNAse-free water and quantified spectrophotometrically at 260–280 nm. RNA (5 μg) was transcribed reversely in a final volume of 20 μL with 1X polymerase chain reaction (PCR) buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 20 mM DTT, 0.5 mM each of 2′-deoxynucleoside-5′-triphosphates (dATP, dGTP, dCTP and dTTP), 40 units Recombinant RNasin ribonuclease inhibitor (Promega, Madison, WI, USA), 200 units M-MLV Reverse Transcriptase (Promega), and 0.5 μg oligo (dT)15 primer (Promega). The reverse transcription (RT) reactions were carried out for 60 min at 42°C and heated to 94°C for 5 min to terminate the RT reaction.

Polymerase chain reaction

Primers used for the amplification of the rat β-actin amplification were: 5′-TTGTAACCAACTGGGACGATA TGG-3′ (sense) and 5′-GATCTTGATCTTCATGGTGCT AGG-3′ (antisense). The primers used for the NAT amplification were: 5′-CAGTGTGACCAGCTCTGTGG-3′ (sense) and 5′-ACCGATGATGAAGGCCACAAGACA-3′ (antisense), and for the HIOMT were: 5′-GG-TAGCTCCGTGTGTGTCTT-3′ (sense) and 5′-AGTGG-CCAGGTTGCGGTAGT-3′ (antisense). The primers used for the melatonin receptor MT1 were 5′-GCCACAGT CTCAAGTATGATAGG-3′ (sense) and 5′-GGTGA-CAAAGTTCCTGAAGTC-3′ (antisense). Each reaction contained 5 μL RT reaction product as template DNA, 1X PCR buffer, 0.4 mM each deoxynucleotide, 2.5 units Taq DNA Polymerase (Promega), 1.5 mM $MgCl_2$, and 1 μM sense and antisense primers. The template was initially denatured for 3 min at 94°C followed by different programs optimized for: β-actin cDNA (30-cycle program with 1 min at 94°C, 1 min at 55°C, 1 min at 72°C), NAT cDNA (35-cycle program with 1 min at 94°C, 2.5 min at 55°C, 1 min at 72°C), HIOMT cDNA (45-cycle program with 2 min at 94°C, 2.5 min at 45°C, 2 min at 72°C), MT1 cDNA (41-cycle program with 2 min at 94°C, 1 min annealing at 55°C, 2 min extension at 72°C). All programs were terminated by 10-minute extension at 72°C. For each PCR run a negative control was systematically added, in which water replaced cDNA. PCR markers were used as size standards.

Southern blot

After amplification, 5 μL PCR reaction were electrophoresed in 2% agarose gel in 1X TAE buffer and visualized by staining with ethidium bromide and UV illumination using DNA molecular weight marker VI DIG-labeled 0.15–2.1 kbp as size marker. Electrophoresed PCR products were transfered to a hy+-nylon membrane with 10X SSC as transfer solution and cross-linked to the nylon membrane using a calibrated UV light source. Blots were prehybridized at 68°C for 3 hr in prehybridization buffer. The hybridization was performed at 60°C overnight in the same prehybridization buffer plus 20 ng DNA/mL of: 329 bp fragment labeled probe for MT1 product (5′-TGA GTGTCAGTGTCCATATCAGGAACACGT-3′), 395 bp fragment for NAT product (5′-CTAGGAACTCTGAGGT CCCAAGAGGCAGAT-3′) and 363 bp fragment for

HIOMT product (5′-ATGTTGAGTGACAGCAGGAGCGACCGCAGT-3′). Thereafter, blots were washed twice for 5 min in 2X SSC/0.1% SDS at room temperature and twice for 6 min in 0.1X SSC/0.1% SDS at 60°C. To detect the hybridization signal, blots were incubated for 30 min in 0.1 M maleic acid/0.15 M NaCl/1% blocking reagent and for 30 min with anti-DIG-AP (anti-digoxigenin conjugated to alkaline phosphatase). Finally, they were washed and incubated in CSPD. Blots were exposed to Kodak X-OMAT AR film (KODAK, Rochester, NY, USA) at room temperature.

NAT activity assay

NAT activity was determined by the method of Champney et al. [26]. Each thymus was homogenated in 300 μL 0.05 M PBS, pH 6.8. Ten microliters of this homogenate were mixed with 10 μL of PBS containing 40 nCi [1-^{14}C] acetylcoenzyme A and 5.6 mM tryptamine. The reaction was carried out for 20 min at 37°C, and was stopped by the addition of 100 μL 0.2 M sodium borate buffer, pH 10, and 1 mL chroloform at 4°C. The N-acetyltryptamine produced was extracted with chloroform and its radioactivity was measured by liquid scintillation spectometry with a beta counter. NAT activity was expressed as nmol N-acetyltryptamine produced/per mg protein per hr. Protein content was measured following the Bradford protocol [27].

HIOMT activity assay

HIOMT activity was determined by the method of Champney et al. [26] by measuring the amount of melatonin formed from N-acetylserotonin and S-adenosyl-L-methionine. Thymus was homogenated in 300 μL 0.05 M PBS, pH 6.8. Forty microliters of this homogenate were mixed with 20 μL 0.05 M PBS, pH 7.9, containing 20 nCi S-[methyl-^{14}C] adenosyl-L-methionine and 3 mM N-acetylserotonin. The reaction was incubated for 20 min at 37°C and was stopped by the addition of 100 μL 0.2 M sodium borate buffer pH 10 and 1 mL chloroform at 4°C. Synthesized melatonin was measured following extraction in 1 mL chloroform and the radioactivity by liquid scintillation spectometry counted with a beta counter. HIOMT activity was expressed as nmol melatonin per mg protein per hr. Protein content was measured following the Bradford protocol [27].

Melatonin determination

Thymus melatonin determinations were measured by HPLC and fluorimetric detection procedure validated in our laboratory. Tissue samples were homogenized in PBS/6.8 + 0.1% ethanol and centrifuged at 3000 g for 10 min. Supernatants (500 μL) were mixed with 1 mL chloroform, shaken for 20 min and centrifuged at 9000 g for 10 min. Aliquots of supernatant were frozen at −80°C for protein analysis [27]. After washing the organic phase twice with 0.05 M carbonate buffer, pH 10.25 was dried. The residue was redissolved in 100 μL HPLC mobile phase and 70 μL were injected into the HPLC system. The samples were injected onto a μBondapack-C18 ODS reversed-phase column (10 μm of particle size; Waters S.A., Barcelona, Spain). The mobile phase consisted of 0.1 M sodium phosphate, 50 mg/L EDTA, and 30% acetonitrile, pH 5.1. The system was run at a flow rate of 0.9 mL/min (Waters 600E pump; Waters S.A.). The fluorescence detector (LS 40; Perkin Elmer Ltd, Buckinghamshire, UK) was set at excitation/emission wavelength of 285/345 nm. The identification of peaks by retention time and their quantification by peak height was performed using a HP 3396 integrator (Hewlett Packard Co, Palo Alto, CA, USA). The detection limit was 10 pg/injection and the inter and intra-assay variation coefficients were less than 7% and 4%, respectively.

Image and data analysis

Ethidium bromide-stained PCR products run on agarose gel were photographed under UV light and the images were scanned. Quantification of the autoradiographic signal was performed using the PC-bas 2.0 processing and analysis program. The relative mRNA abundance of target genes was calculated as the ratio of the target gene autoradiographic signal density to that of β-actin.

Results relating to enzyme activities and melatonin content were expressed as the mean ± S.E. Statistical analysis were performed using one-way ANOVA followed by Bonferroni's test.

Results

The two key enzymes of melatonin synthesis, NAT and HIOMT, as well as melatonin content were analyzed to demonstrate melatonin synthesis in developing rat thymus. As shown in Figs 1A and 2A, there is expression of NAT and HIOMT mRNA from fetal day 18 (F18) through postnatal days 7, 16, 60 (P7, P16, and P60). Moreover, the expression of the enzyme mRNA correlates with a progressive increase of both thymic NAT (Fig. 1C) and HIOMT (Fig. 2C) activities, both enzymes exhibiting the maximal activity at postnatal day 60 (P60). Results obtained, for both thymic mRNA expression and enzyme activity studies, showed both parameters were clearly increased in animals killed during daytime when compared with values obtained in those killed at night (Figs 1 and 2).

Melatonin content was also studied in the developing thymus. Results in Fig. 3 show that melatonin is present in the thymus early in its development. Apparently, specific melatonin content decreases with age and is higher at night in F18 and P7, while exhibiting lower values at night in P16 and P60. It is not possible to explain the discrepancy between melatonin content results and the expression/activity of NAT and HIOMT. However, as the presence of melatonin in thymus can be explained by a double origin, i.e. melatonin synthesized in the thymus and melatonin synthesized in the pineal gland, we performed experiments in which melatonin of pineal origin was absent.

The effect of pinealectomy on melatonin content in thymus is shown in Fig. 4. The results demonstrate that pinealectomized rats have clearly increased the thymic melatonin content, showing a threefold increase, when compared with sham-pinealectomized animals. These data suggest that either pineal melatonin could be inhibiting

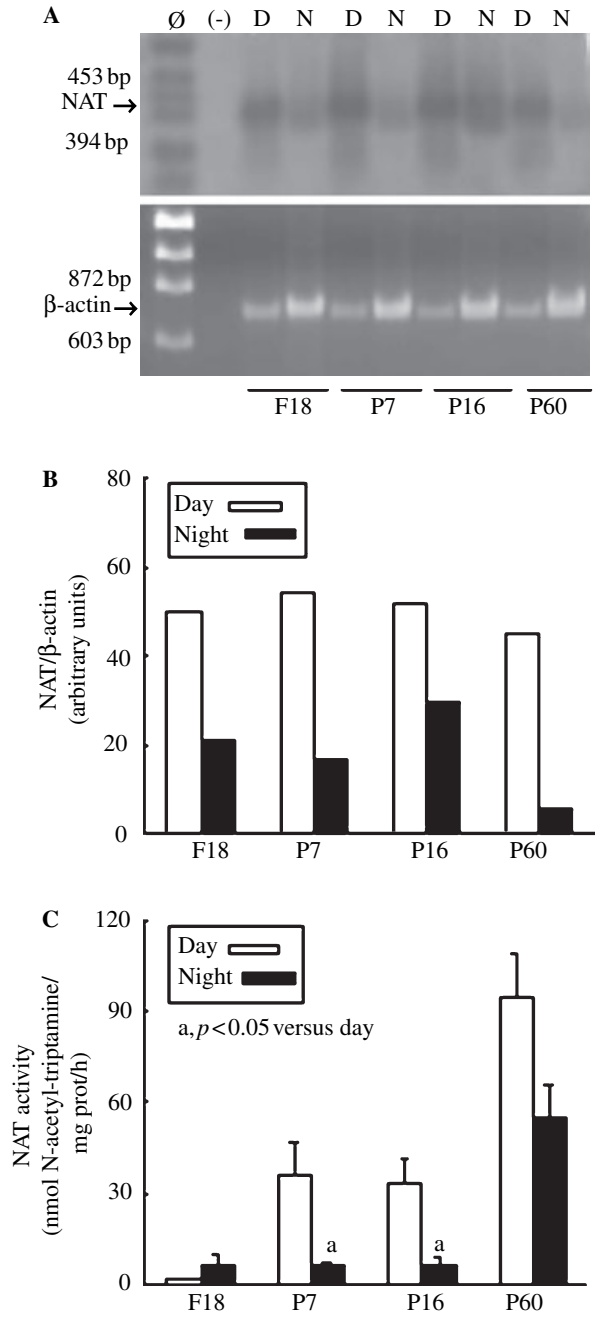

Fig. 1. Temporal patterns of NAT mRNA expression and NAT activity levels in developing rat thymus. (**A**) Southern Blot analysis of NAT and RT-PCR of β-actin of RNA extracted from thymuses of rats killed on fetal day 18 (F18) and postnatal days 7, 16 and 60 (P7, P16, and P60) either during the day (D) or at night (N). A control reaction with no cDNA (−) as well as weight markers (Φ) were also included. (B) Ratio between NAT mRNA expression and β-actin mRNA expression. Values are the mean of three experiments. (C) NAT activity in thymuses of rats killed at the indicated ages either during the daytime or at night. Values are the mean ± S.E. of six experiments.

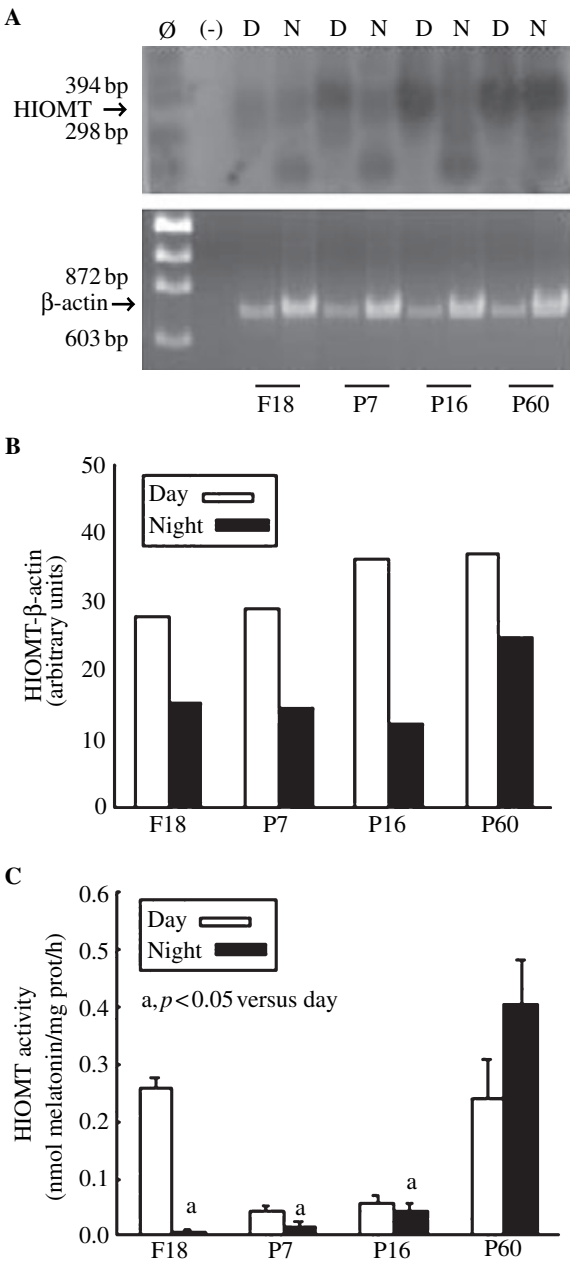

Fig. 2. Temporal patterns of HIOMT mRNA expression and HIOMT activity levels in developing rat thymus. (A) Southern blot analysis of HIOMT and RT-PCR of β-actin of RNA extracted from thymuses of rats killed on fetal day 18 (F18) and postnatal days 7, 16 and 60 (P7, P16, and P60) either during the day (D) or at night (N). A control reaction with no cDNA (−) as well as weight markers (Φ) were also included. (B) Ratio between HIOMT mRNA expression and β-actin mRNA expression. Values are the means of three experiments. (C) HIOMT activity in thymuses of rats killed at the indicated ages either during the daytime or at night. Values are the means ± S.E. of six experiments.

thymic synthesis of melatonin and/or, when pineal-synthesized melatonin is absent, the thymus could show a compensatory rise in melatonin production.

In order to evaluate whether the developing thymus was able to respond to melatonin, melatonin-membrane receptor MT1 was analyzed at fetal day 18 (F18) and postnatal days 7, 16, and 60 (Fig. 5). As shown in Fig. 5(A,B) mRNA expression for MT1 receptor was clearly detected at 18 days of fetal life and was similar at all stages of development.

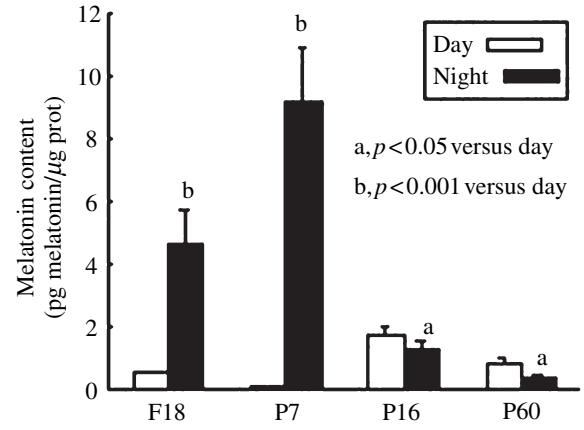

Fig. 3. Melatonin content in the thymus of rats from fetal to the adult stage. Rats were killed at midday (white bars) and at night (black bars) at different stages of the development. Thymuses are removed for melatonin determinations. Results are the means ± S.E. of eight animals.

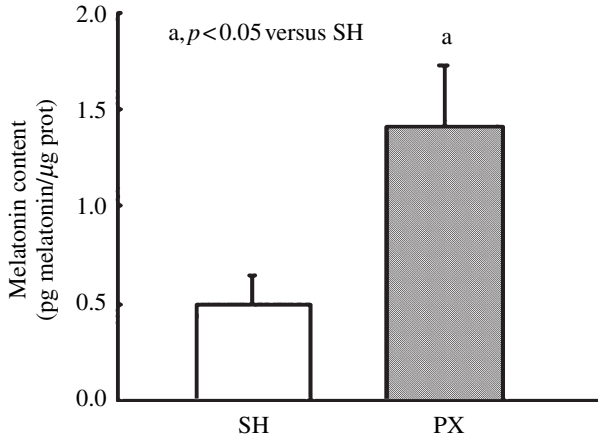

Fig. 4. Effect of pinealectomy on thymus melatonin content. Newborn rats were pinealectomized (Px) or sham-pinealectomized (Sh) and killed at night on postnatal day 16 for measurement of thymus melatonin. Values are the means ± S.E. of eight animals.

Indeed, at night, the expression of mRNA MT1 receptor was lower when compared with the expression during the day.

While trying to determine whether the decrease in MT1 expression related to the downregulation phenomena described for melatonin receptors, the expression of mRNA MT1 receptor in pinealectomized animals was studied. Results indicate (Fig. 6) that, after pinealectomy, there is a decrease in MT1 expression, which correlates with an increase in thymic melatonin content (Fig. 4).

Discussion

The results of this study clearly demonstrate melatonin synthesis in the developing rat thymus, as we found expression and activity of the two key melatonin biosynthetic enzymes and detected significant melatonin

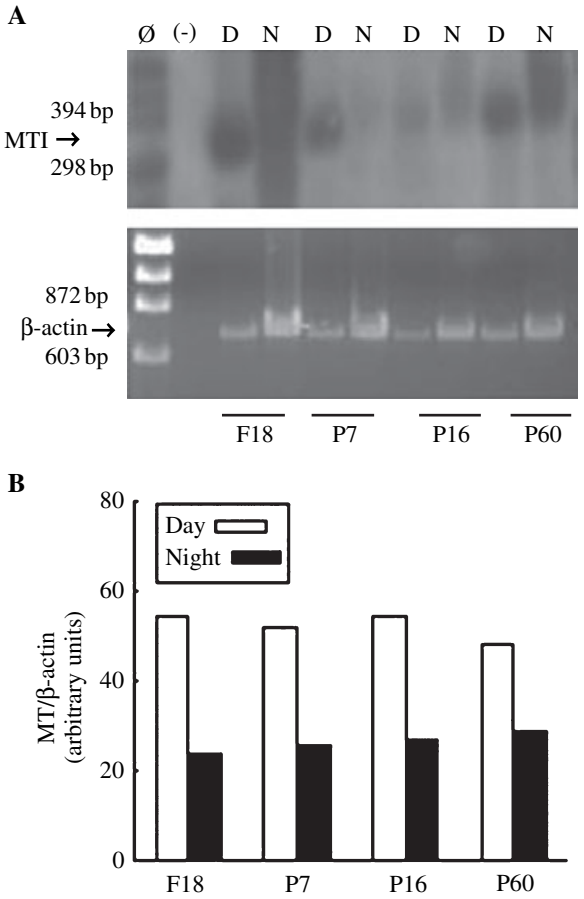

Fig. 5. Developmental patterns of MT1 melatonin receptor expression in rat thymus. (A) Southern blot analysis of MT1 and RT-PCR of β-actin of RNA from thymuses of rats killed on fetal day 18 (F18) and postnatal days 7, 16, and 60 (P7, P16, and P60) either during the daytime (D) or at night (N). A control reaction with no cDNA (−) as well as molecular weight markers (Φ) were also included. (B) Ratio between MT1 mRNA expression and β-actin mRNA expression. Values are the means of 3 experiments.

levels. Extrapineal melatonin synthesis has been described in many other tissues including retina, gastrointestinal tract, reproductive organs, skin, and platelets [7]. Likewise, melatonin production has been documented in the rat immune system [8–10] and human lymphocytes [11].

In this paper, we detect melatonin production in thymus as from 18 fetal day by analyzing both NAT and HIOMT expression and activity, and melatonin content. An even earlier onset of melatonin synthesis is possible although earlier stages were not studied. Therefore, at least, both enzymes were present and functional in the thymus at F18. At this time, NAT and HIOMT transcripts and HIOMT activity showed significant day/night differences with increased diurnal values. Moreover, we observed a similar NAT and HIOMT temporal expression pattern without a great variation between fetal and postnatal days (P7 and P16). As expected, NAT and HIOMT activity followed the same developmental appearance, showing a diurnal rhythm with higher levels in daytime than at night. Both enzyme activities reached the highest values at the adult stage but did not show significant diurnal differences.

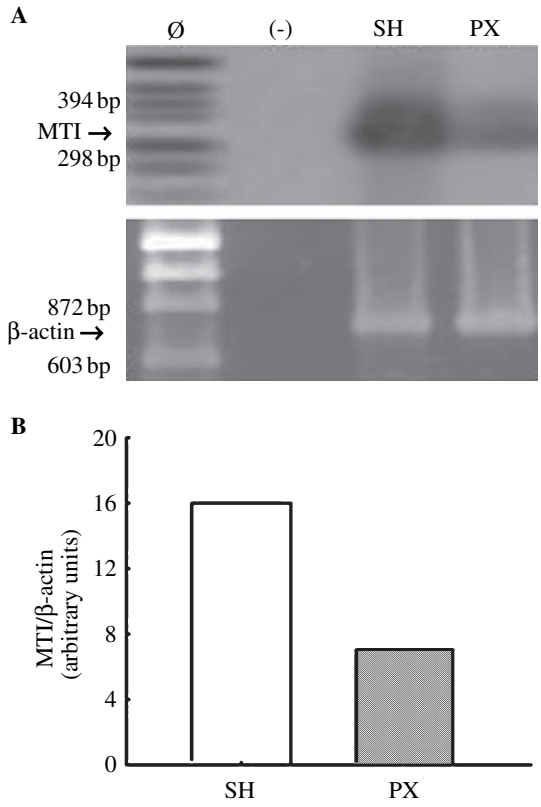

Fig. 6. Effect of pinealectomy on thymus MT1 melatonin receptor mRNA expression. Newborn rats were pinealectomized (Px) or sham-pinealectomized (Sh) and killed on postnatal day 16. (A) Southern blot analysis of MT1 mRNA and RT-PCR analysis of β-actin. A control reaction with no cDNA (−) as well as molecular weight markers (Φ) were also included. (B) Ratio between MT1 mRNA expression and β-actin mRNA expression. Values are the means of 3 experiments.

For the first time, we demonstrate melatonin contents in thymus at all stages studied but, unexpectedly, the diurnal rhythm of melatonin content did not follow the same pattern as the enzymes. In this way, we found increased nocturnal melatonin content at F18 and P7 when the enzyme activities were low, especially HIOMT activity. This discrepancy could be explained if thymic melatonin was derived from, at least, two pathways: the maternal and the fetal origin. A number of works have described the existence of placental transfer of melatonin that could result in a daily maternally generated melatonin rhythm in the fetus. Furthermore, this maternally derived melatonin reaches all tissues in nonhuman primates and neonate rats [28]. Consequently, we believe that maternally derived melatonin may be taken up by the thymus to contribute to these high melatonin levels during the night. Moreover, there are several reports concerning melatonin synthesis in fetal tissues, such as pineal and retina [29, 30]. The thymus could also contribute although its melatonin production is low at this time. However, in P16 and P60 rats, when the pineal is mature and shows a circadian rhythm with increased levels at night [5], melatonin content in thymus showed decreased nocturnal values. This observation supports the hypothesis that pineal melatonin production may inhibit melatonin synthesis in thymus as we found different results depending on whether the pineal is functional or not.

Many studies have used pinealectomy to study the effect of pineal melatonin on melatonin production of extrapineal tissues such as the rat brain [31, 32] or rat Harderian gland [33], and in the retina [33, 34], where an increase in retinal melatonin content was demonstrated suggesting that the biosynthesis of this indole in the retina may be modulated via feedback from pineal melatonin. We studied melatonin content in sham-operated and pinealectomized rats to demonstrate our hypothesis of the pineal regulation of melatonin synthesis in the thymus, as the nocturnal peak of melatonin reaches its maximum and maturation of pineal gland takes place in the third postnatal week [5]. After pinealectomy, the thymic melatonin content was higher than in sham-operated rats, as indicated by threefold rise, therefore the thymus may not take up pineal melatonin and increase its synthesis.

Finally, we investigated the developmental appearance of MT1 receptor in developing thymus as well as its time- and age-related expression patterns. We found MT1 expression with similar transcript levels at fetal stage when compared with postnatal and adult values. Thus, the fetal thymus is capable of responding to a melatonin signal as early as day 18 of gestation. The presence of fetal melatonin-binding sites has been demonstrated in the ovine fetus [35], in the central nervous system and the pituitary of fetal rat [36] as in the suprachiasmatic nucleus of the fetal Syriam hamster [37]. Furthermore, the concentration of melatonin receptor sites in the rat pituitary was shown to be highest in 20-day-old fetuses and gradually decreased in the course of postnatal life [38].

In conclusion, rat thymus in late stages of fetal development, as well as in postnatal and adult life, synthesizes melatonin. The thymus-derived melatonin may be negatively regulated by melatonin of pineal origin and, apparently, can act on its own site of synthesis interacting with its melatonin receptors. Although additional experiments are required to better understand the role of pineal on the thymus and the physiologic relevance of melatonin on newborn rats, the results support the hypothesis that melatonin can act as an intra-, auto-, or paracrine substance, particularly, in the thymus [11, 39] and in the immune system in general.

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