

Research Article

Melatonin biosynthesis in the thymus of humans and rats

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Received 30 September 2006; received after revision 30 December 2006; accepted 15 February 2007
Online First 2 March 2007

Abstract. Melatonin is an indoleamine widely distributed in the evolution that shows a great functional versatility, playing an important role as a transmitter of photoperiodic information and exhibiting antioxidant, oncostatic, anti-aging and immunomodulatory properties. In vertebrates, this molecule is produced by the pineal gland and other extrapineal sites. The present study was carried out to investigate the presence of melatonin in thymus and the possibility of an endogenous melatonin synthesis in this organ, in which T cells are matured. In this work, we demon-

strate in humans and rats that thymus contains melatonin, expresses the mRNAs encoding *N*-acetyltransferase and hydroxyindol-*O*-methyltransferase, the two key enzymes of the melatonin synthesis, and has this biosynthetic machinery activated. In addition, rat thymocytes cultured for 24 h exhibited high levels of melatonin. The results presented here suggest that human and rat thymuses are able to synthesize melatonin, which could have intracrine, autocrine and paracrine functions.

Keywords. Extrapineal melatonin, thymus, T cells, NAT, HIOMT, neuroimmunomodulation.

Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is an indoleamine derived from the amino acid tryptophan, with serotonin being the most important intermediate product. Its biosynthetic pathway involves four well-defined intracellular enzymatic steps [1] catalyzed by tryptophan hydroxylase (TPH), aromatic amino acid decarboxylase (AADC), serotonin *N*-acetyltransferase (arylalkylamine-*N*-acetyltransferase; AA-NAT) and hydroxyindole-*O*-methyltransferase (HIOMT).

Melatonin was first discovered and isolated in the pineal gland by Lerner in 1958 [2]. Pineal melatonin is synthesized and released into blood at night, and this allows the regulation of circadian rhythms and seasonal changes in vertebrates [3, 4]. Besides playing an important role as a transmitter of photoperiodic information, melatonin shows antioxidant [5–7], oncostatic [8–10], anti-aging [11, 12] and immunomodulatory [13–15] properties, as well as many other physiological functions [16]. This great functional versatility is reflected in its wide distribution within phylogenetically distant organisms, from bacteria to humans [10, 17].

Extrapineal melatonin was revealed in the 1970s when, after the removal of the pineal gland, melatonin

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was still present in blood plasma and urine of laboratory animals (total daily excretion of melatonin in pinealectomized rats was about 20% of control), indicating a significant extrapineal synthesis [18]. Since then, the search for extrapineal sources of melatonin has awakened a great interest.

As soon as highly sensitive antibodies to indolealkylamines became available [19], melatonin was identified in many extrapineal tissues [20]. This presence does not necessarily indicate an endogenous synthesis, because the high lipid and water solubility of melatonin [21, 22] permits its transfer through biological membranes, and, therefore, the indolemine is found throughout the organism in all cells and cellular organelles, including the nucleus [10]. At this moment, extrapineal melatonin synthesis has been demonstrated only in eye structures [10, 23, 24], the Harderian gland [25], gut [26], skin [27], inner ear [28], ovary [29, 30], testes [31] and the immune system [32–35]. It is hypothesized that, while pineal melatonin seems to act as a typical hormone, reaching widely spread target cells through the bloodstream, extrapineal melatonin may play intracrine, autocrine and paracrine key roles [20, 36].

In the last three decades, numerous studies have shown the relationship between melatonin/pineal gland and immune system [37]. In particular, in the case of thymus, it is known that pinealectomy causes a reduction in the weight of thymus and its structural disorganization [38, 39], and these are reversed by administering melatonin [40]. The aging involution of thymus is also partly reversed by ingestion of melatonin [41]. This molecule also increases the production of some thymic peptides [42], and prevents apoptosis in thymus [43]. Presence of melatonin receptors in thymus completes the evidence of this relationship [44–46].

Recently, our group has described an endogenous melatonin biosynthesis in rat thymus during development [47]. In the present work, we probe deeper into melatonin production in thymus of adult rats, carrying out additional studies, as well as demonstrating, for the first time, a melatonin synthesis in thymus of humans.

Materials and methods

Human samples. Fragments of human thymus and blood samples were obtained from patients undergoing a routine heart operation. These patients, nine men and two women aged between 21 and 76 years, underwent the surgery between 10:00 and 12:00 h. The thymus fragments that hindered the access to the heart in the operation were removed, briefly rinsed in normal saline solution and stored at -80°C until their use for melatonin measurement, RNA extraction and enzyme activity assays. Blood samples were collected in tubes with EDTA and centrifuged for 20 min at 1500 g at 15°C . Plasma samples were obtained and frozen at -20°C until

melatonin determinations by ELISA were performed.

The human experimental procedures were reviewed and approved by the Virgen del Rocío Hospital Ethical Committee.

Rat tissues. Male rats of the Wistar strain (*Rattus norvegicus*), born and bred in our animal facilities, were housed under controlled photoperiods (12 h light:12 h darkness. Lights were turned off daily from 20:00 to 08:00 h) and maintained with food and water *ad libitum*.

Two-month-old rats were killed by decapitation between 12:00 and 14:00 h and blood samples, thymuses and pineals were obtained. Blood samples were centrifuged for 20 min at 1500 g at 15°C , and sera were collected and frozen at -20°C until melatonin determinations by ELISA were carried out. Pineals and thymuses were quickly removed and briefly rinsed in normal saline solution. Pineals were frozen on solid CO_2 and stored at -80°C until their use for RNA extraction. Half of each thymus was frozen on solid CO_2 and stored at -80°C until its use for melatonin measurement and RNA extraction. The other half was used to obtain thymocytes and stroma. These were frozen on solid CO_2 and stored at -80°C until their use for RNA extraction.

For the enzyme activity determinations, 1- and 2-month-old rats were used. These were killed by decapitation between 12:00 and 14:00 h and thymuses and pineals were quickly removed and briefly rinsed in normal saline solution. Half of each thymus was used to obtain thymocytes and stroma, while the other half was homogenized. The fresh samples were used immediately in the enzyme activity assays.

The experiments were conducted in accordance with the Spanish Government Guide and the European Community Guide for Animal Care.

Preparation of rat thymocytes. Half of each rat thymus from freshly killed animals was immersed in cold saline 0.9% (w/v) and cut into small pieces with scissors. Cells were isolated by pressing the suspension of the freshly minced organ in saline through a nylon mesh. The liberated thymocytes were pelleted by centrifugation at 400 g for 10 min, treated with erythrocyte lysis buffer (saline 0.2%) for about 25 s and washed three times in saline 0.9% before use, at 400 g for 10 min. The remaining tissue in the nylon mesh after filtering was the thymus stroma. Stroma and thymocytes were frozen or freshly utilized.

Cell culture. Thymocytes were resuspended in RPMI 1640 medium with 25 mM HEPES (GIBCO-BRL, Life Technologies Ltd., Paisley, Scotland) supplemented with 1 mM L-glutamine, 50 U/mL penicillin, 50 $\mu\text{g}/\text{mL}$ streptomycin (mixture from Sigma-Aldrich Co. Ltd., Dorset, UK) and 10% fetal calf serum (BioWhittaker, Boehringer Ingelheim, Verviers, Belgium), and were cultured for 24 h at a density of 5×10^6 cells/mL in flasks (EasYFlasks™ 25 filter. Nunc A/S, Denmark) at 37°C under a humidified 5% CO_2 atmosphere. Cell viability was determined by trypan blue exclusion.

After incubation, the culture supernatants were collected and stored at -20°C for melatonin determinations.

RNA extraction and first-strand cDNA synthesis. Total RNA was extracted from the samples by a modification of Chomczynski and Sacchi's method [48], using TriPure Isolation Reagent (Roche, Mannheim, Germany) as denaturing solution and appropriate chloroform volume. After cell lysis and RNA extraction, RNA was precipitated with isopropanol, and the pellet was washed in 75% ethanol. The RNA samples were recovered by centrifugation at 7500 g for 5 min and then dried. Each RNA pellet was dissolved in 100 or 150 μL RNase-free water and quantified spectrophotometrically at 260 nm.

After this, 5 μg RNA was reverse transcribed in a final volume of 40 μL to obtain single-stranded cDNA using the following method: 5 μg RNA were denatured in 19 μL RNase-free water at 85°C for 10 min, and then rapidly chilled on ice. Then, 21 μL of a mixture formed by 1 \times reverse transcription (RT) buffer, 20 mM dithiothreitol (DTT), 2'-deoxyribonucleoside-5'-triphosphates (0.5 mM of each dNTP: dATP, dGTP, dCTP and dTTP), 40 U recombinant RNasin ribonuclease inhibitor, 0.5 μg oligo(dT)₁₅ primer and 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV RT) were added (all reagents from Promega, Madison, WI, USA).

The RT reaction was carried out for 60 min at 42°C and heated at 94°C for 5 min to terminate the RT reaction.

Polymerase chain reaction. The cDNA was amplified in a reaction containing 5 µL of RT product as template DNA, 1× PCR buffer, MgCl₂ in an appropriate concentration, 0.4 mM each deoxynucleotide, 2.5 U ECOTAQ DNA polymerase (Ecogen, Barcelona, Spain) and 0.2 µM sense and antisense primers of housekeeping gene (β -actin) and 1 µM sense and antisense primers of the gene under study, in a final volume of 25 µL. The template was initially denatured for 3 min at 94°C followed by different programs optimized for: rat NAT cDNA (40-cycle program with 1 min of denaturation at 94°C, 2 min annealing at 65°C, 1 min extension at 72°C; with 1.5 mM MgCl₂); rat HIOMT cDNA (40-cycle program with 1 min at 94°C, 2 min at 60°C, 2 min at 72°C; with 1 mM MgCl₂); human NAT cDNA (35-cycle program with 1 min at 94°C, 1 min at 55°C, 1 min at 72°C; with 2 mM MgCl₂), and human HIOMT cDNA (40-cycle program with 1 min at 94°C, 2 min at 60°C, 2 min at 72°C; with 2 mM MgCl₂). All programs were terminated by an extension of 10 min at 72°C. For each run of PCR a negative control was systematically added, in which water replaced cDNA. In addition, the ubiquitously expressed β -actin mRNA was used to monitor the quality of RNAs and the efficiency of the RT and the PCR. β -Actin amplification was performed simultaneously with the other genes. The primers used for the β -actin PCR were: 5'-TTG TAA CCA ACT GGG ACG ATA TGG-3' (sense) and 5'-GAT CTT GAT CTT CAT GGT GCT AGG-3' (antisense), obtaining a PCR product of 746 bp. Primers used for the rat NAT amplification were: 5'-CAG TGT GAC CAG CTC TGT GGT GG-3' (exon 1) and 5'-CCG ATG ATG AAG GCC ACA AGA CA-3' (exon 3; PCR product of 394 bp) and for the rat HIOMT were: 5'-GGT AGC TCC GTG TGT GTC TT-3' (exon 6) and 5'-AGT GGC CAG GTT GCG GTA GT-3' (exon 8; PCR product of 363 bp). Primers for the human NAT PCR were: 5'-TGC CAG TGA GTT TCG CTG CCT C-3' (exon 2) and 5'-ACC TGT GCA GCG TCA GTG ACT C-3' (exon 4; PCR product of 242 bp), and for the human HIOMT were: 5'-CAT GAC TGG GCA GAC GGA AA-3' (exon 8) and 5'-GTT AGT TCC AGG TCA CAA GAA ACA GTT-3' (exon 9; PCR product of 301 bp).

Southern blot. After amplification, 5 µL PCR reaction product were separated by agarose gel electrophoresis (2% w/v) in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and visualized by staining with ethidium bromide (0.5 µg/mL) and UV illumination, using a 100-bp ladder as DNA size marker (Biotools, Madrid, Spain). Together with these, DNA Molecular Weight Marker VI digoxigenin (DIG)-labeled 0.15–2.1 kb (Roche) was also added as a Southern size marker. Electrophoresed PCR products were transferred to a positively charged nylon transfer membrane (Amersham Biosciences, Amersham, UK) with 10× SSC as transfer solution and cross-linked to the nylon membrane using a calibrated UV light source. Blots were prehybridized at 68°C for 2 h in prehybridization buffer (5× SSC, 0.1% *N*-laurylsarcosyl, 0.02% SDS, 1% blocking reagent). The hybridization was performed at 60°C overnight in the same prehybridization buffer plus 20 ng DNA/mL labeled probe. Thereafter, blots were washed twice for 5 min in 2× SSC/0.1% SDS at room temperature and twice for 10 min in 0.1× 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-DIG conjugated to alkaline phosphatase). Finally, they were washed and incubated with the chemiluminescent substrate CSPD (Roche). Blots were exposed to Kodak X-OMAT AR film (Rochester, NY, USA) at room temperature and later developed.

The probes used in this study were: 5'-CTA GGA ACT CTG AGG TCC CAA GAG GCA GAT-3' and 5'-GTG ACA GCT CTG GAC ACA GGG TGA GGA AGT-3' for rat NAT product, 5'-ATG TTG AGT GAC AGC AGG AGC GAC CGC AGT-3' and 5'-ATC AGG GAG ATC AAA GAC ACA CAC GGA GCT-3' for rat HIOMT product, 5'-GCT CGA TCT CAA AGG CGC TGA CAG CGT CCT-3' for human NAT product, 5'-CTC TCC AGC AGG TGT GAG CAC TTT CCG TCT-3' for human HIOMT product. Probes were labeled with DIG with an oligonucleotide tailing kit (Roche). **NAT activity assay.** NAT activity was determined by the method of

Champney et al. [49]. Each sample (pineal, thymus, thymocytes or stroma) was homogenated at 4°C in 0.05 M phosphate-buffered saline buffer (PBS), pH 6.8, using a cell sonicator (Sonics and Materials Inc., Danbury CT, USA). Ten microliters of this homogenate were mixed with 10 µL PBS containing 40 nCi [¹⁴C]acetylcoenzyme A and 5.6 mM tryptamine. In the case of cells, the homogenate was previously centrifuged (3 min, 16 000 g), and 10 µL supernatant was utilized for assay activity. 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 chloroform at 4°C. The *N*-acetyltryptamine produced was extracted with chloroform and its radioactivity was measured by liquid scintillation spectrometry with a beta counter. NAT activity was expressed as nmol *N*-acetyltryptamine produced/mg protein/h. Protein content was measured following the Bradford protocol [50].

HIOMT activity assay. HIOMT activity was determined by the method of Champney et al. [49] by measuring the amount of melatonin formed from *N*-acetylserotonin and *S*-adenosyl-L-methionine. Each sample was homogenated in 0.05 M PBS, pH 6.8. Forty microliters of this homogenate, or homogenate supernatant in the case of thymocytes, were mixed with 20 µL 0.05 M PBS, pH 7.9, containing 20 nCi *S*-[methyl-¹⁴C]adenosyl-L-methionine and 3 mM *N*-acetylserotonin. The reaction was incubated for 30 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 spectrometry counted with a beta counter. HIOMT activity was expressed as pmol or nmol melatonin/mg protein/h. Protein content was measured following the Bradford protocol [50].

Melatonin determinations. Melatonin levels were determined by a competitive enzyme immunoassay kit (Immuno Biological Laboratories, Hamburg, Germany) according to manufacturer's instructions. The volume of each thymus fragment was previously measured, to refer to volume the melatonin measures. Thymus fragments were homogenated in PBS with 0.1% ethanol in a sonicator (Sonics and Materials Inc., Danbury CT, USA) and centrifuged at 3000 g for 10 min. Thymus homogenate supernatants, serum and culture supernatants were used for melatonin determinations.

Melatonin from 500 µL of the samples, standards and controls was extracted (90–100% yield recovery) using C18 reversed-phase columns (IBL-Hamburg, Germany) and methanol elution. The dried extracts (after evaporating methanol) were stored at –20°C for up to 48 h. Melatonin levels were measured in duplicate using 96-well microtiter plates coated with captured antibody goat anti-rabbit Ig. Each microtiter plate was filled either with 50 µL blank reagent, extracted calibrators, extracted samples or extracted standard solutions (containing 0, 3, 10, 30, 100 or 300 pg/mL melatonin). Then, 50 µL melatonin biotin and 50 µL rabbit-antiserum were added into each well, shaken carefully, sealed with adhesive foil and incubated overnight (14–20 h) at 2–8°C. After washing three times with 250 µL diluted assay buffer, 150 µL anti-biotin conjugate to alkaline phosphatase was added into each well and incubated for 2 h at room temperature. The reaction was developed using *p*-nitrophenyl phosphate and optical densities were determined at 450 nm in an automatic microplate reader. The sensitivity of the melatonin assay was 3.0 pg/mL. Both the intra- and inter-assay coefficients of variation (CV) were less than 10%. **Statistical data analysis.** Data were statistically analyzed using a paired Mann-Whitney test. Results are expressed as mean ± standard error of the mean (SEM).

Results

Melatonin biosynthesis in rat thymus. Melatonin content in rat thymus was measured at midday by ELISA and compared with melatonin content in rat

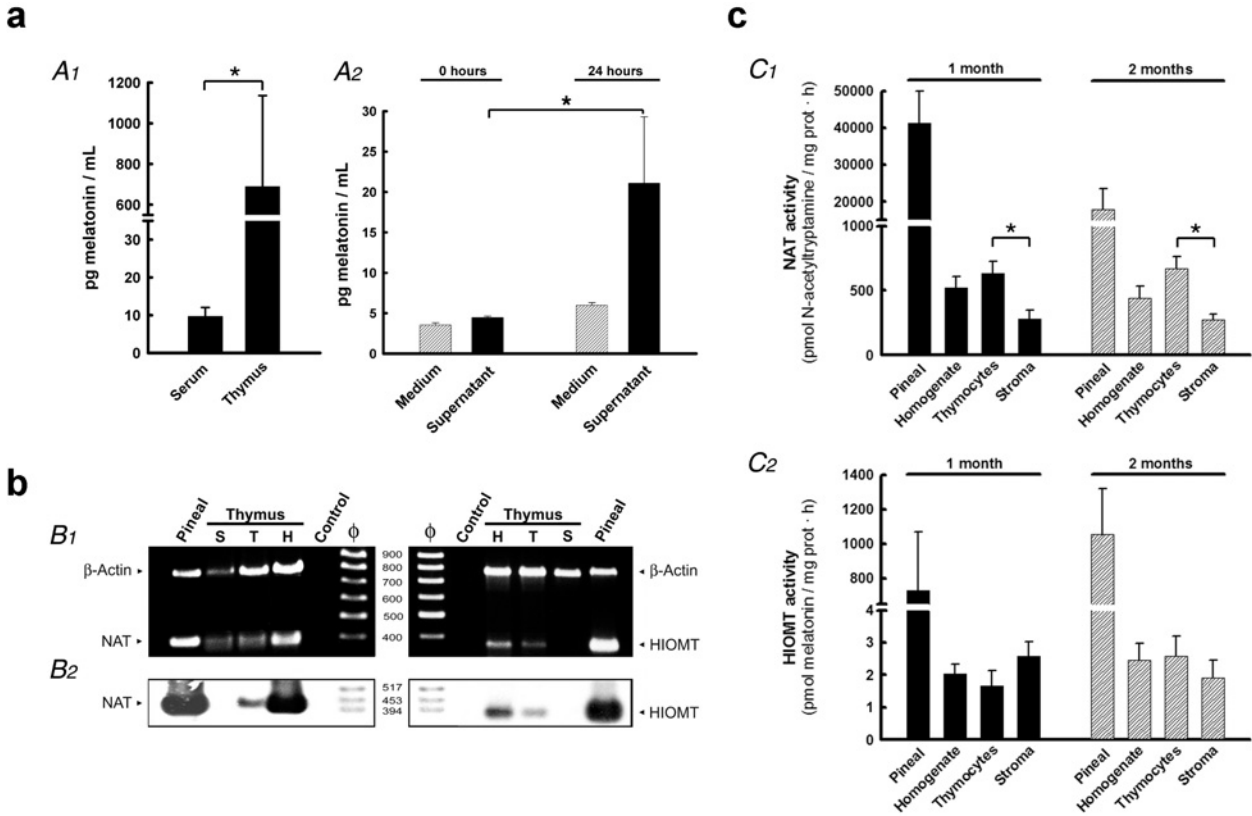


Figure 1. Melatonin biosynthesis in rat thymus. (a) Melatonin measurement by ELISA. (A₁) Melatonin concentration in serum and thymus homogenate of rats. Data are expressed as mean ± SEM of eight experiments performed in duplicate. Significant differences between serum and thymus concentrations were observed; * $p < 0.01$. (A₂) Melatonin measurement in rat thymocytes cultures. Cells were cultured for 24 h. Melatonin was measured in supernatant at 0 and 24 h, finding a significant difference; * $p < 0.01$. To eliminate any contribution to the melatonin content from the culture medium itself, flasks with culture medium without cells underwent the same process as the cell cultures. Values are the means of three experiments performed in duplicate. (b) Presence of the two key enzymes involved in the melatonin synthesis (NAT and HIOMT). (B₁) RT-PCR analysis of NAT (left) and HIOMT (right) mRNA expression in thymus homogenate (H), isolated thymocytes (T) and thymic stroma (S). mRNA expression in pineal homogenate was used as positive control. β -Actin was amplified as a housekeeping gene. PCR molecular size marker (ϕ) was used (100-bp ladder), and PCR reaction without cDNA substrate was used as PCR control. (B₂) Southern blot hybridization of the PCR products with the DIG-labeled specific probes. Southern molecular size marker was used (DNA Molecular Weight Marker VI DIG-labeled 0.15–2.1 kb). (c) NAT and HIOMT activities. Activities of NAT (C₁) and HIOMT (C₂) were measured in thymus homogenate, isolated thymocytes, and thymic stroma of 1- and 2-month-old rats. The pineal glands were used as positive control of NAT and HIOMT activities. Data are expressed as mean ± SEM of seven experiments performed in duplicate. Significant differences between stroma and thymocytes were observed for NAT activity; * $p < 0.01$. No significant differences were observed between measurements of 1- and 2-month-old rats.

serum, using samples from 2-month-old rats. The results, expressed as pg melatonin/mL, showed a large amount of melatonin in thymus, two orders in magnitude more than in serum (Fig. 1a, A₁). Moreover, when thymocytes were cultured for 24 h, a great increase of melatonin levels in culture supernatants throughout this time was observed (Fig. 1a, A₂). To eliminate any contribution to the melatonin content from the culture medium itself, flasks with culture medium without cells underwent the same process as the cell cultures.

After that, several experiments were carried out to confirm the endogenous synthesis in rat thymus. First, we verified the presence of the biosynthetic machinery in rat thymus, studying, by RT-PCR (Fig. 1b, B₁)

and Southern blot (Fig. 1b, B₂), the expression of the genes that code the two key enzymes of melatonin synthesis, AA-NAT and HIOMT. AA-NAT is the rate-limiting enzyme in melatonin synthesis, and HIOMT is the final enzyme of the biosynthetic pathway. The electrophoresis of PCR products (394 bp for AA-NAT and 363 bp for HIOMT) and the Southern blot showed that AA-NAT and HIOMT are expressed in thymus homogenate and thymocytes. In stroma the expression is lower for the AA-NAT (Fig. 1b, left) and seems not to exist for the HIOMT (Fig. 1b, right). Pineal homogenate was used as a positive control. For each run of PCR a negative control was systematically added, in which water replaced cDNA. The β -actin was used as housekeeping gene, and its amplification

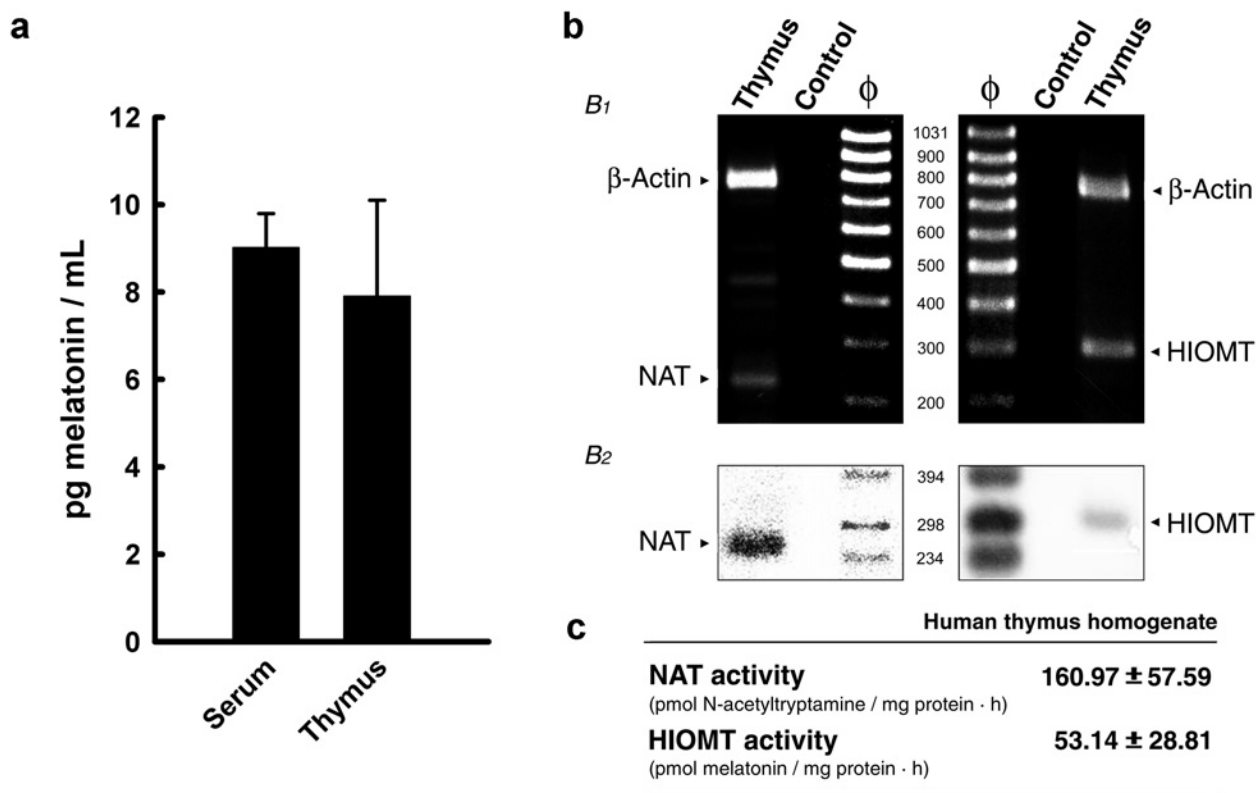


Figure 2. Melatonin production in human thymus. (a) Melatonin measurements in serum and thymus homogenate. Melatonin was measured by ELISA. Data are expressed as mean \pm SEM of five experiments performed in duplicate. (b) The presence of NAT and HIOMT. (B₁) RT-PCR analysis of AA-NAT (left) and HIOMT (right) mRNA expression in thymus homogenate. Pineal homogenate was used as positive control. β -Actin was amplified as a housekeeping gene. PCR molecular size marker (ϕ) was used (100-bp ladder), and PCR reaction without cDNA substrate was used as PCR control. (B₂) Southern blot hybridization of the PCR products with the DIG-labeled specific probes. Southern molecular size marker was used (DNA Molecular Weight Marker VI DIG-labeled 0.15–2.1 kb). (c) NAT and HIOMT activities in thymus homogenate. Values are the means of six experiments performed in duplicate.

was performed simultaneously with the other genes (PCR product of 746 bp).

We then checked if this enzymatic machinery was active by measuring the enzymatic activity of NAT and HIOMT. Both enzymes are active in 1- and 2-month-old rats (Fig. 1c). These experiments showed that NAT activity mainly resides in thymocytes (Fig. 1c, C₁), while HIOMT activity does not present significant differences between thymocytes and stroma (Fig. 1c, C₂). Pineal homogenate was used as a positive control.

Melatonin production in human thymus. Following the same procedure as in rats, melatonin content in human thymus and serum was measured by ELISA. We observed a high level of melatonin in thymus homogenate (Fig. 2a). Data are expressed as pg melatonin/mL. No differences were found between thymus and serum.

Moreover, the presence of the biosynthetic machinery in the human thymus was investigated. To determine the expression of the AA-NAT and HIOMT mRNA, we subjected mRNA from human thymus homogenate to RT-PCR analysis using specific primers for

both genes. Electrophoresis of PCR products (Fig. 2b, B₁) revealed the presence of the expected bands for the AA-NAT (242 bp) and for the HIOMT (301 bp). No band was obtained from reaction in which cDNA was omitted. To test the quality of RNA and efficiency of RT, we subjected all samples to the RT-PCR of housekeeping gene β -actin. The β -actin primers amplified the expected 746-bp product in all samples. The Southern blot analysis performed with DIG-labeled NAT (Fig. 2b, B₂, left) and HIOMT (Fig. 2b, B₂, right) probes confirmed that both genes are expressed in thymus homogenate.

The activity study of both enzymes revealed NAT and HIOMT activities in thymus homogenate (Fig. 2c).

Discussion

For years it was believed that vertebrates only synthesized melatonin in the pineal gland. Now it is known that the Harderian gland, gut, skin, inner ear, ovary, testes, some structures of eye, some elements of

the immune system and possibly other tissues can synthesize this molecule [10, 24, 32, 34, 51].

Melatonin production has been reported in cells and cell lines of the immune system in several cases [32–35, 52]. The results shown in the present work suggest an endogenous melatonin biosynthesis in thymus of humans and rats. We have found melatonin in abundance and the expression and activity of the two key melatonin biosynthetic enzymes, NAT and HIOMT, have been detected.

The studies in rats revealed a large amount of melatonin in thymus homogenate, two orders in magnitude more than in serum (Fig. 1a, A₁). This is in accordance with the levels found in bone marrow [32], the other primary lymphatic organ. This result may indicate that either the thymus accumulates circulating melatonin or synthesizes melatonin, or both.

Thymocytes were then cultured for 24 h, and the culture supernatants also exhibited high levels of melatonin after this time (Fig. 1a, A₂). To eliminate a contribution to the melatonin content from the culture medium itself, flasks with culture medium without cells underwent the same process as the cell cultures. In addition, to eliminate the possibility that melatonin found in the culture supernatants resulted from a simple diffusion from intracellular space instead of a *de novo* synthesis, we washed thymocytes with saline several times in the cell isolation procedure. Furthermore, prior to culture experiments, the thymocytes melatonin content was negligible when measured after the isolation procedure (results not shown).

RT-PCR studies showed that NAT and HIOMT expression was found principally in thymocytes (Fig. 1b, B₂). NAT activity also resides mainly in thymocytes (Fig. 1c, C₁). No differences were found between thymocytes and stroma HIOMT activities (Fig. 1c, C₂). As NAT is the rate-limiting enzyme in the melatonin biosynthetic pathway, we hypothesize that the greatest amount of melatonin in thymus is synthesized by thymocytes.

Our findings, although a bloodstream melatonin retention cannot completely be ruled out, demonstrate an endogenous biosynthesis in rat thymus, as the AA-NAT and HIOMT mRNAs are expressed and give rise to active enzymes, and thymocytes in culture exhibit high levels of melatonin. Moreover, a recent study of our group showed that pinealectomized rats have a clearly increased thymic melatonin content [47], evidencing an endogenous synthesis, and suggesting that either pineal melatonin could be inhibiting thymic synthesis of melatonin and/or, when pineal-synthesized melatonin is absent, the thymus could show a compensatory rise in melatonin production. Experiments in humans showed a high level of

melatonin in thymus homogenate (Fig. 2a). No differences were found between thymus and serum. As the measurements referred to volume, we suppose that this fact is due to the great fat content in adult human thymus. Expression (Fig. 2b) and activity (Fig. 2c) of the two key enzymes of the melatonin biosynthetic pathway, NAT and HIOMT, were also demonstrated. All these results lead us to suppose an endogenous melatonin synthesis in human thymus.

In general, while pineal melatonin seems to act as a typical hormone in the organism, exerting an endocrine action reaching its target cells through the bloodstream, extrapineal melatonin may play intracrine, autocrine and paracrine key roles in those tissues where it is synthesized [23, 37, 53]. In this way, the resulting melatonin action on a tissue is the sum of the circulating melatonin action, most of which is of pineal origin, plus the endogenous melatonin action (Fig. 3). Furthermore, the endogenous melatonin synthesis levels seem to be regulated by the circulating levels of melatonin [32, 54, 55].

Numerous investigations have highlighted the importance of melatonin in the immune system and, especially, in thymus. Most of them have studied the role of the pineal gland and pineal melatonin. Therefore, circadian variation of pineal melatonin seems to be correlated with circadian variation in cell activity and proliferation in the immune system [13] and the loss of this molecule with age has been associated with the senescence of immune system [56–58].

The most remarkable change that occurs with age is thymic involution, which involves a gradual atrophy and replacement by fat. This physiological involution may be accelerated by endogenous or exogenous factors: hormonal disorders, severe stress, dietary impairment, bacterial endotoxins, ionizing radiation and treatments with corticoids, antitumor drugs or adrenergic drugs. Under these stimuli the thymus rapidly diminishes in size, due to massive death of cortical thymocytes and their removal by macrophages [13, 59, 60]. Thymus involution involves a gradual regression in the size, weight and cellularity of the thymus, principally by an increase in cell death and a decrease in proliferation. Three different types of cell death have been morphologically described in rat thymus: necrosis, apoptosis and clustered cell death [61]. They all increase with aging, but the most significant rise is found in thymic cortex apoptosis. Furthermore, the number of mitoses diminishes, especially in the cortex [59], where the most immature thymocytes are localized [62]. In addition, and probably as a consequence of these, an increase in the percentage of double-negative thymocytes (DN) and a decrease in the double-positive thymocytes (DP) are observed with age [63] inside the thymus. This agrees

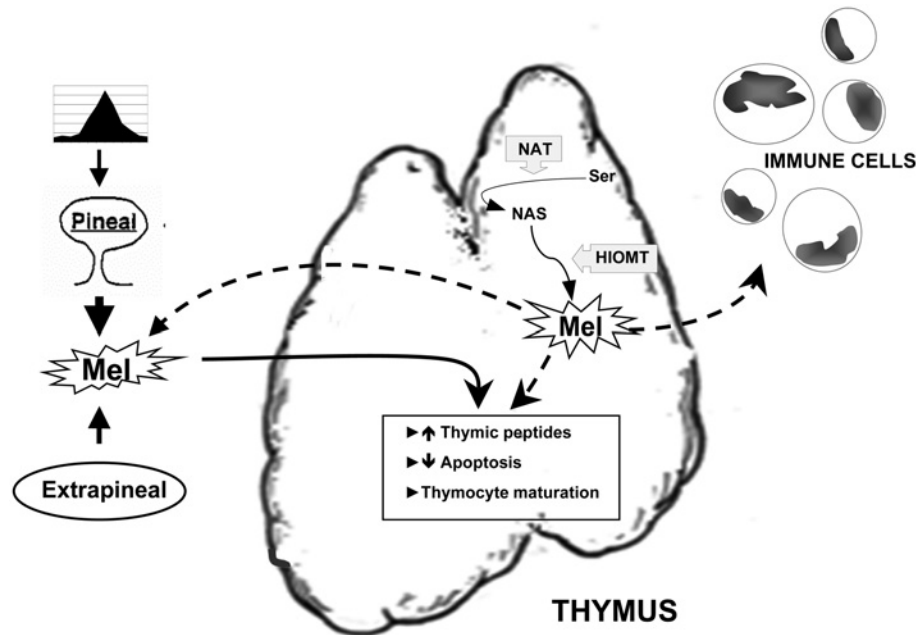


Figure 3. Hypothetical scheme of the melatonin action on thymus. The resulting melatonin action on thymus would be the sum of the circulating melatonin action, most of which has pineal origin, plus the endogenous melatonin action. Melatonin could modulate thymocytes maturation by affecting cytokine production, increasing some thymic peptides production and preventing cell oxidative damage and apoptosis.

with other observations that suggest that one or more developmental blocks of the most immature thymocyte subsets occur with age [64], thus limiting differentiative transitions within the DN population and decreasing progression to DP. The loss of thymus functionality consequently leads to a decreased production of thymic peptides and some cytokines (like IL-7 [56] and IL-2 [63]) and to a weakening of cell-mediated immunity [41].

The aging involution of thymus has been observed to be reversed by administration of melatonin [41]. Moreover, pinealectomy is known to cause a reduction in the weight of thymus and its structural disorganization [38, 39], which is also reversed by administering melatonin [40].

The ways by which melatonin reverses thymic involution seem to be linked with its anti-apoptotic properties [43] and its capacity to modulate the proliferation [65]. The mechanisms whereby melatonin influences apoptosis have not yet been clarified, although different options have been suggested. Melatonin has been found to inhibit glucocorticoid- and hydroxyl radical-induced thymocytes apoptosis, through its anti-oxidative effects and inhibiting glucocorticoid receptor nuclear translocation in thymocytes [41, 66, 67]. On the other hand, this molecule can protect the thymus by modulating zinc levels [68–70], necessary for the active form of thymulin and other functions [71], and by increasing some thymic peptides production [42]. It has been reported that zinc, thymulin and prothymosin alpha also have proliferative and anti-apoptotic properties [71, 72].

Presence of melatonin receptors in the thymus

indicates other mechanisms by which melatonin can exert its actions [44–46]. Therefore, it is very possible that melatonin modulates maturation of thymocytes: a. Through membrane receptors. Expression of MT1 and MT2 receptors has been detected in thymus [44], and MT1 in all different thymocyte subpopulations [45]. Although melatonin functions mediated by these membrane receptors in thymus are unknown, MT1 and MT2 have been shown to influence cytokine production and cell proliferation in other immune system cells [14, 73].

b. By means of nuclear receptors [44, 46]. The most immature DN thymocytes proliferate and produce IL-2 upon stimulation. Subsequently, when DN cells differentiate into more mature DP cells, they lose the capacity for both proliferation and IL-2 production. Not until these cells become fully mature single-positive thymocytes (SP) do they regain this capacity [74]. The melatonin ROR γ receptor, in the thymus-specific variant ROR γ -t [75, 76], seems to be implicated in different thymopoiesis processes. The expression of ROR γ -t appears to be tightly regulated in developing thymocytes, because only DP thymocytes express it [77]. It has been proposed that ROR γ -t inhibits proliferation and IL-2 production in DP subpopulation [78]. At the DP stage, thymocytes face positive and negative selection, and it is very important that neither of these selection events should be accompanied by cell division or cytokine production. The phenotype of ROR γ -t-deficient mice shows increased apoptosis in DP thymocytes [79, 80], changes in DN to DP transition and increased susceptibility to thymic lymphomas [81, 82].

A differential melatonin localization has been suggested in the thymocytes subpopulations [83], each one with a particular cytokine and peptide production [74, 84], proliferation and selection levels [62], indicating a possible different melatonin role along the thymocytes maturation and, probably, along the age. This could explain why melatonin presents both proliferative [65] and anti-proliferative properties [85] in thymus. We hypothesize that melatonin proliferative action on the small DN subpopulation would be barely perceptible, as DP thymocytes, with a melatonin anti-proliferative actions [78, 85], represent most of thymic cells. Only when DN thymocytes accumulate in the thymus involution can the melatonin proliferative action on these cells be appreciated [65]. On the other hand, melatonin seems to act in intrathymic positive selection, inducing the sensitivity of thymocytes for negative selection to undergo apoptosis and, at the same time, preserving the thymocytes from apoptosis for positive selection and maturation [71].

In conclusion, the results shown here demonstrate an endogenous melatonin synthesis in thymus of humans and rats. This endogenous melatonin, together with pineal melatonin and other hormonal and non-hormonal agents, could modulate and regulate the thymus function and homeostasis [56, 71]. Further experiments are necessary to determine the function of endogenous melatonin in the thymus.

Acknowledgements. This work was supported by grants from the Spanish Government (PI06/0091 and P/2004/504). The authors thank Mr. John Brown for correcting the English language.

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