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Molecular, Biological, Physiological and Clinical Aspects of Melatonin

Extrapineal melatonin: analysis of its subcellular distribution and daily fluctuations

Abstract: We studied the subcellular levels of melatonin in cerebral cortex and liver of rats under several conditions. The results show that melatonin levels in the cell membrane, cytosol, nucleus, and mitochondrion vary over a 24-hr cycle, although these variations do not exhibit circadian rhythms. The cell membrane has the highest concentration of melatonin followed by mitochondria, nucleus, and cytosol. Pinealectomy significantly increased the content of melatonin in all subcellular compartments, whereas luzindole treatment had little effect on melatonin levels. Administration of 10 mg/kg bw melatonin to sham-pinealectomized, pinealectomized, or continuous light-exposed rats increased the content of melatonin in all subcellular compartments. Melatonin in doses ranging from 40 to 200 mg/kg bw increased in a dose-dependent manner the accumulation of melatonin on cell membrane and cytosol, although the accumulations were 10 times greater in the former than in the latter. Melatonin levels in the nucleus and mitochondria reached saturation with a dose of 40 mg/kg bw; higher doses of injected melatonin did not further cause additional accumulation of melatonin in these organelles. The results suggest some control of extrapineal accumulation or extrapineal production of melatonin and support the existence of regulatory mechanisms in cellular organelles, which prevent the intracellular equilibration of the indolamine. Seemingly, different concentrations of melatonin can be maintained in different subcellular compartments. The data also seem to support a requirement of high doses of melatonin to obtain therapeutic effects. Together, these results add information that assists in explaining the physiology and pharmacology of melatonin.

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Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine, aMT) is a highly preserved molecule produced by most of the living organisms, including bacteria, macroalgae, plants, invertebrates, and mammals [1–3]. First isolated from the pineal gland [4], melatonin is now believed to be produced in a variety of other organs. Melatonin is synthesized from the amino acid tryptophan with the production of serotonin as an intermediate compound. The synthesis of melatonin is controlled by two main enzymes: *N*-acetyltransferase (NAT) and hydroxyindole-*O*-methyltransferase (HIOMT) (now known as acetylserotonin methyl transferase). Although NAT has been considered the rate-limiting enzyme in the synthesis of melatonin, there are now evidence supporting that HIOMT regulates its production under some circumstances [5].

The synthesis of pineal melatonin is strictly controlled by the light/dark cycle. The regulation is mediated through a multisynaptic pathway starting with the ganglion cells of the retina whose axons form the retinohypothalamic tract which projects to the suprachiasmatic nucleus. The information arrives at the pineal via peripheral postganglionic sympathetic fibers [6]. During darkness, norepinephrine released from the postganglionic sympathetic fibers binds to and activates both β- and α-adrenergic receptors in the membrane of the pinealocyte, increasing intracellular cAMP which in turn induces the transcription of NAT mRNA [7, 8]. During the day, light blunts the norepinephrine release to the pinealocyte, inhibiting NAT and melatonin production. This photoperiodic control of the pineal metabolic activity creates a day/night cycle in the melatonin synthesis as also reflected in the circadian rhythm of the blood melatonin levels. The day/night oscillation of melatonin in the blood represents a peripheral clock involved in the regulation of biological rhythms such as the sleep–wake cycle [9] and seasonal rhythms [10]. Besides its chronobiotic properties, melatonin is also a potent antioxidant and anti-inflammatory molecule [11–15].

Owing to its lipophilicity, melatonin crosses all biological barriers, acting on all subcellular compartments to prevent oxidative/nitrosative damage. Of note, melatonin is particularly important to maintain mitochondrial homeostasis [16–18]. The presence and effects of melatonin in the cell were initially thought to be related to its uptake from the blood. However, it has become apparent that many cells may have the enzymatic machinery also to produce melatonin [19]. Early indications of alternative sources of melatonin were shown by the presence of HIOMT in both the retina [20] and Harderian glands [21]. Melatonin was then identified in enterochromaffin cells of human intestinal mucosa, with concentrations of melatonin in the gastrointestinal tract more than 400 times higher than in the pineal gland, and 10–100 times higher than in plasma [22, 23]. Then, melatonin was found in the plasma and urine of pinealectomized rats [24]. Recently, the analysis of NAT and HIOMT mRNA expression using PCR revealed the existence of possible melatonin synthesis in a wide variety of tissues including thymus, spleen, heart, muscle, liver, stomach, intestine, placenta, testis, cerebral cortex, and striatum [19]. Most recently, melatonin in extrapineal tissue homogenates of thymus, spleen, liver, kidney, and heart has been measured [25]. The levels of melatonin in these tissues seem to be higher than the concentrations of indoleamine. Moreover, melatonin content in peripheral organs decreases with age, to a similar extent as the pineal melatonin production [26, 27].

Although preliminary studies have evaluated the subcellular distribution of extrapineal melatonin [28], the mechanism(s) that regulates its levels in subcellular organelle and whether its concentration varies with time of day are yet unknown. Thus, we examined the subcellular concentrations of melatonin and its daily distribution in rat liver and brain tissues in both normal and pinealectomized animals. Moreover, the participation of melatonin membrane receptors in the intracellular content of the indoleamine and the dose-dependent effects of melatonin on its subcellular accumulation were also analyzed.

Materials and methods

Reagents

Melatonin, EDTA-Na_2, mannitol, EGTA, fatty acid-free BSA, and dithiothreitol (DTT) were purchased from Sigma-Aldrich (Madrid, Spain). Trichloromethane, sodium phosphate, acetonitrile, sucrose, and Tris–HCl were purchased from Panreac (Barcelona, Spain). Luzindole was purchased from Tocris (Tocris Bioscience, Ellisville, MO, USA). All others reagents were of the highest purity available.

Animals and treatments

Three-month-old male Wistar rats were obtained from Harlan (Barcelona, Spain). They were housed in clear plastic cages and maintained under controlled photoperiods of 12:12-hr light/dark cycle (lights on at 7 hr) at 22 ± 1°C and they were given regular chow and tap water, under the supervision of veterinarians. All experiments were performed according to the Spanish Government Guide and the European Community Guide for animal care. Another group of animals was maintained at a continuous light regime for at least 7 days. The animals were grouped (six rats per group) as follows: (a) controls; (b) sham-pinealectomized (SPx) animals; (c) pinealectomized (Px) rats; and (d) continuous light-exposed (CL) animals; these rats were kept under CL for 5 days before tissue collections. The final group of rats (group e) was treated with the melatonin membrane receptor blocker, luzindole (LZ).

To study the circadian variations in the subcellular distribution of melatonin, groups of SPx and Px animals were sacrificed at 08:00, 12:00, 16:00, 20:00, 24:00, 02:00, 04:00, and 06:00 hr under a 12:12 light/dark cycle. To test whether the pineal gland affects the subcellular distribution of exogenous melatonin, SPx, Px, and CL animals were i.p. injected with 10 mg/kg bw melatonin at 08:00 or 10:00 hr and sacrificed at 12:00 hr, i.e., 2 and 4 hr after the melatonin injection. Two additional groups of SPx animals were subjected to the same experimental protocol except for an injection of LZ at 07:30 hr. To study the dose-dependent effects of melatonin on the subcellular distribution of the indoleamine, groups of control rats were i.p. injected with 0, 10, 40, 100, or 200 mg/kg bw at 08:00 hr and sacrificed at 12:00 hr, i.e., 4 hr later. At night, the animals were sacrificed under red light.

Melatonin was dissolved in 20% 1,2-propanediol and injected intraperitoneally. LZ was dissolved in a water/ethanol 1:2 (v/v) and injected intraperitoneally at a dose of 4.5 mg/kg, 30 min prior melatonin administration. The pharmacokinetic study of plasma melatonin after i.p. administration of 10 mg/kg bw showed a half-life of 27 min (Fig. 1). Pinealectomy was performed after i.p administration of 1 mL/kg bw equithesin anesthesia [29], and the animals were studied 5 days later. The greater reduced circulating melatonin levels after Px or CL are shown in the Fig. 2. At the end of experiments, rats were anesthetized with chloroform, and blood samples were

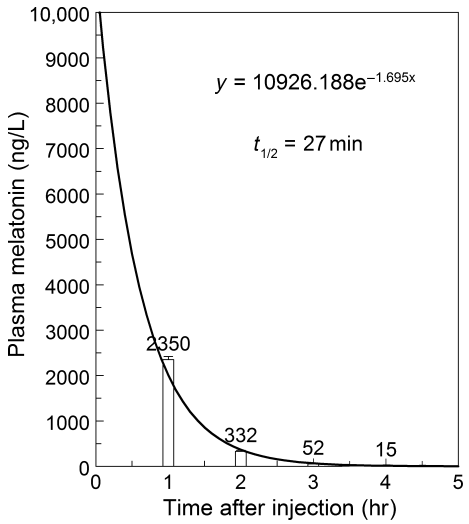

Fig. 1. Pharmacokinetics of plasma melatonin after an intraperitoneal injection of 10 mg/kg bw.

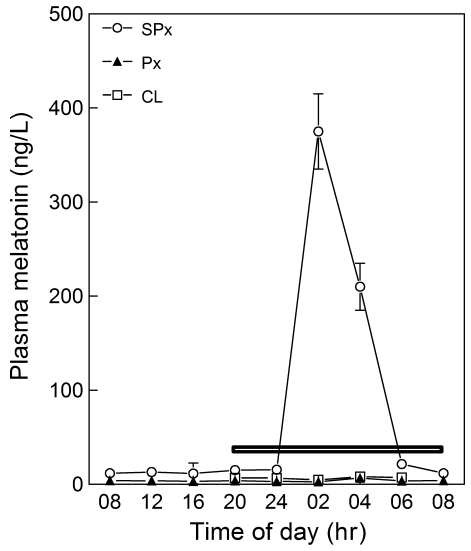

Fig. 2. Levels of plasma melatonin in sham-pinealectomized (SPx), pinealectomized (Px), and continuous light-exposed rats. Black bar indicates the dark period.

obtained via cardiac puncture. Then, the brain and liver were immediately removed, washed in cold saline, and stored at −80°C until their use. These procedures were performed under red light in those animals sacrificed at night. Blood samples were centrifuged at 1500 g for 10 min at 4°C, and plasma was frozen at −80°C for the determination of melatonin.

Isolation of pure mitochondria, nuclei, and cytosol from cerebral cortex and liver

Brain cerebral cortexes and livers were washed with the corresponding extraction buffers A (10 mM Tris–HCl, 0.32 M sucrose, 1 mM EDTA-K_2, pH 7.4) and B (5 mM Hepes, 250 mM mannitol, 0.5 mM EGTA-K_2, and 0.1% fatty acid-free BSA, pH 7.4), respectively. The tissues were minced with scissors, homogenized with the respective extraction buffer at 800 rpm with a Teflon pestle, and filtered.

Cerebral cortex was processed as follows: the filtered homogenate was centrifuged at 1330 g for 3 min at 4°C, and the pellet was resuspended in 0.5 mL extraction buffer A and centrifuged again in the same conditions. The pellet of this second centrifugation is used for nuclei preparation. Meanwhile, the supernatants of both centrifugations were mixed and centrifuged at 21,200 g for 10 min at 4°C. The supernatant of this step was used for cytosol preparation, whereas the mitochondrial pellet was suspended in 0.85 mL extraction buffer A containing 15% Percoll, poured into ultracentrifuge tubes containing a Percoll gradient formed by 1 mL 40% Percoll and 1 mL 23% Percoll in buffer A, and centrifuged at 22,500 g for 8 min at 4°C. Pure mitochondria, corresponding to the fraction between 23% and 40% Percoll, were collected, washed with buffer A, and centrifuged at 16,700 g for 10 min at 4°C. The pellet was washed again and centrifuged at 6900 g for 10 min at 4°C to remove any excess of Percoll, and the final pellet containing pure brain mitochondria was frozen at −80°C.

Rat liver was processed as follows: the homogenate was centrifuged at 600 g for 5 min at 4°C (twice). The pellet of the second centrifugation is used for nuclei preparation. Meanwhile, the supernatants of both centrifugations were mixed and centrifuged at 10,300 g for 10 min at 4°C. The supernatant of this step was used for cytosol preparation, whereas the mitochondrial pellets were suspended in 0.5 mL buffer B and poured into ultracentrifuge tubes containing 1.4 mL buffer C (225 mM mannitol, 1 mM EGTA, 25 mM Hepes, and 0.1% BSA) and 0.6 mL Percoll. The mixture was centrifuged at 105,000 g for 30 min at 4°C (Optima L-90K ultracentrifuge; Beckman Coulter, Madrid, Spain), and the fraction corresponding to the pure mitochondrial fraction was collected, washed with buffer B, and centrifuged at 10,300 g for 10 min at 4°C (Avanti 30; Beckman Coulter). The pellets were washed and centrifuged again at 6300 g for 10 min at 4°C to remove the Percoll and frozen at −80°C.

The pellets resulting from the second centrifugation during cerebral cortex or liver processing were resuspended in 3 mL buffer D (10 mM Tris–HCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM DTT, 0.3 M sucrose, 0.15% Triton X-100, pH 8); these were poured into tubes containing 3 mL buffer D plus 0.4 M sucrose and centrifuged at 2500 g for 10 min at 4°C. The resulting nuclear pellet was gently resuspended, without vortexing, in 1 mL of buffer A, centrifuged once again, and frozen at −80°C.

The supernatants resulting from the third centrifugation were used to obtain the cytosol fraction. These supernatants were centrifuged at 100,000 g for 1 hr (Optima L-90K ultracentrifuge; Beckman Coulter) and stored at −80°C.

Isolation of pure cell membranes from cerebral cortex and liver

Pieces from cerebral cortex and liver were washed in 1 mM $NaHCO_3$ buffer, pH 8, minced with scissors, homogenized in the same buffer at 800 rpm with a Teflon pestle, diluted to 8.1 mL with $NaHCO_3$, and filtered. The filtered homogenates were centrifuged at 1500 g for 15 min at 4°C (Beckman Avanti 30 centrifuge, Madrid, Spain), resuspended in 1.8 mL 10 mM Tris–HCl buffer, pH 7.6, containing 71% (w/v) sucrose, and stirred for 15 min. An aliquot of this suspension (0.45 mL) was transferred to ultracentrifuge tubes, at which were successively added 0.53 mL of 10 mM Tris–HCl, pH 7.6, containing 53% (w/v) sucrose, 0.53 mL of 10 mM Tris–HCl, pH 7.6, containing 42% (w/v) sucrose and, finally, 0.53 mL of a 0.25 M sucrose solution. These tubes were centrifuged at 100,000 g for 1 hr at 4°C (Optima L-90K ultracentrifuge; Beckman Coulter), to obtain the pure membrane fraction corresponding to the band between 42% and 53%, which was frozen at −80°C [30].

Determination of melatonin by HPLC

Melatonin in plasma and subcellular fractions was determined by HPLC with fluorescence detection following a method described elsewhere [31]. For plasma, 500 μL was extracted with 1 mL trichloromethane. The mixture was vortexed for 1 min at 1400 rpm and then centrifuged for

1 min at 5000 g. Aqueous phase was removed, and the organic phase was washed thrice with 500 μL 50 mM $NaHCO_3$, pH 10.25. Finally, 500 μL of sample was evaporated to dryness (Speed Vac System; Fisher Scientific, Madrid, Spain) for 33 min (SPD 2010 SpeedVac System; Fisher Scientific) at a vacuum pressure of 5.1 Torr, and the dry extracts obtained were frozen at −80°C until melatonin assay. On the day of the assay, dry extracts were resuspended in 100 μL of mobile phase consisting of 100 mM sodium phosphate, 0.1 mM EDTA, and 25% acetonitrile.

Subcellular frozen samples from cerebral cortex and liver were thawed and sonicated in PBS, pH 7.4, and centrifuged at 3000 g for 10 min at 4°C. Aliquots of the supernatants were frozen at −80°C for protein determination or mixed (500 μL) with 1 mL chloroform, shaken for 20 min, and centrifuged at 9000 g for 10 min at 4°C. The organic phase was washed twice with 0.05 M Na_2CO_3 buffer, pH 10.25, and 500 μL of the samples was evaporated to dryness in a SPD 2010 SpeedVac System (Fisher Scientific). The residue was then dissolved in 100 μL of mobile phase.

Plasma and tissue content of melatonin was then measured by HPLC (Shimadzu Europe GmbH, Duisburg, Germany) with a 150 × 4.5 mm Waters Sunfire C18 5 μm column (Waters Chromatography, Barcelona, Spain). After stabilizing the column with the mobile phase, samples (20 μL) were injected onto the HPLC system at a 1-mL/min flow rate, with 5-fluorotryptamine as internal standard, and the fluorescence of melatonin was measured in a fluorescence detector (Shimadzu RF-10A XL fluorescence detector) with an excitation and emission wavelength of 285 and 345 nm, respectively. Retention time was 8.9 min. A standard curve for melatonin was constructed with 4.45, 8.9, 17.9, 35.9, 71.6, and 143.2 ng/L, and the concentration of melatonin in the samples was calculated according to the peak area. Melatonin levels were expressed in nanogram per liter. 5-Fluorotryptamine was used as an internal standard [32]. Protein levels were measured by Bradford method [33].

Statistical analysis

Data are expressed as the means ± S.E.M. of six determinations. 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). The level of statistical significance was taken as $P < 0.05$.

Results

Daily changes in the subcellular concentration of melatonin in the cerebral cortex are shown in Fig. 3. Although cosinor analysis did not reveal a significant circadian rhythm of melatonin, its levels oscillated over the 24-hr period. In mitochondria, SPx animals showed the highest melatonin levels throughout the 24-hr period. This was followed by membranes and nuclei, whereas the cytosol content of melatonin was the lowest. In general, Px produced an increase in melatonin content in these fractions, mainly in membranes ($P < 0.001$), with lower rises in mitochondria and nuclei. In liver, melatonin levels also did not exhibit a circadian rhythm, and they showed a different pattern of change (Fig. 4). In this tissue, the content of melatonin was higher in membrane than in mitochondria or nuclei, both of which had similar levels; the lowest concentration of melatonin was found in the cytosol. Px also increased the subcellular concentrations of melatonin in liver, mainly in membranes, cytosol, and nuclei ($P < 0.001$), and to a minor extent, in mitochondrion. Mean hepatic levels of melatonin over a 24-hr period in the different subcellular fractions are shown in Table 1. It is seen that the overall

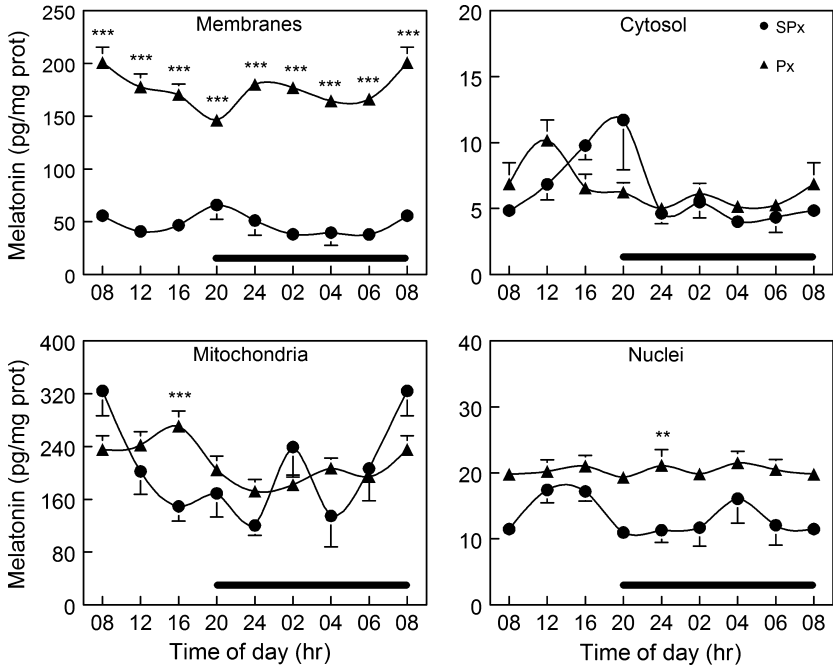

Fig. 3. Daily changes in the melatonin levels of membranes, cytosol, mitochondria, and nuclei of rat cerebral cortex of sham-pinealectomized (SPx) and Px rats. Animals were maintained in a 12:12-hr cycle and sacrificed at the indicated hours. Black bar indicates the dark period. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus SPx.

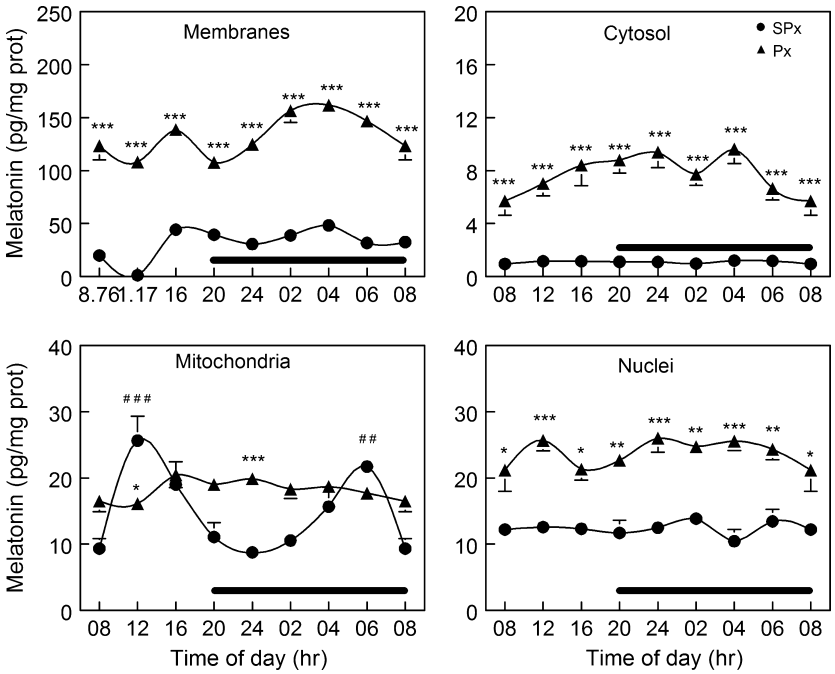

Fig. 4. Daily changes in the melatonin content of membranes, cytosol, mitochondria, and nuclei of rat liver of sham-pinealectomized (SPx) and Px rats. Animals were maintained in a 12:12-hr cycle and sacrificed at the indicated hours. Black bar indicates the dark period. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ versus SPx.

Table 1. Mean melatonin concentrations over a 24-hr cycle in the subcellular fractions studied

Tissue	SPx	Px
Brain		
Membranes	48.01 ± 3.27	176.09 ± 5.77*
Cytosol	6.27 ± 0.9	6.46 ± 0.52
Mitochondria	207 ± 25.3	216 ± 10.6
Nuclei	13.31 ± 0.91	20.31 ± 0.25*
Liver		
Membranes	37.76 ± 2.09	132.38 ± 6.58*
Cytosol	1.09 ± 0.03	7.67 ± 0.5*
Mitochondria	14.57 ± 2.08	18.14 ± 0.52
Nuclei	12.36 ± 0.32	23.64 ± 0.68*

SPx, sham-pinealectomy; Px, pinealectomy.
Data are expressed as means ± S.E.M. $*P < 0.001$ versus SPx.

melatonin content significantly increased in membrane and nuclei in cerebral cortex and in all subcellular fractions in liver after Px. The largest increase in melatonin owing to Px was seen in liver cell membranes.

Fig. 5 shows the changes in the subcellular melatonin levels in the cerebral cortex induced by different manipulations. In all cases, the individual bars represent the levels of melatonin measured at 12:00 hr, i.e., 2 hr after vehicle or melatonin administration. Px ($P < 0.001$) and, to a lesser extent, CL ($P < 0.01$), increased melatonin levels in membranes, but not in the other subcellular fractions in vehicle-treated rats. After the administration of 10 mg/kg bw melatonin, melatonin values increased significantly in SPx, CL, and, specially, in Px rats ($P < 0.001$). Pretreatment with LZ did not alter melatonin levels in Px rats. Similar changes after melatonin treatment were found in the cytosol. Interestingly, mitochondria and nuclei behaved differently. Neither Px nor CL influenced the levels of melatonin in these fractions in vehicle-treated animals. Treatment with melatonin, however, increased its levels at the same extent in SPx, Px, CL, and LZ groups ($P < 0.001$). In the case of liver (Fig. 6), Px and CL increased the melatonin levels in membranes and cytosol, as in the cerebral cortex, whereas Px also elevated nuclear melatonin levels ($P < 0.05$). In these fractions, melatonin administration (10 mg/kg bw) also increased its levels in all groups of rats ($P < 0.001$), whereas LZ pretreatment did not modify the effects of melatonin administration. In general, changes in cytosolic melatonin levels after its administration were similar to those described for the cerebral cortex cytosol. Changes in melatonin in liver mitochondria and nuclei were almost identical to those detected in the same brain subcellular fractions, i.e., melatonin treatment induced parallel rises in these fractions in SPx, Px, CL, and LZ groups ($P < 0.001$). A similar change was obtained in all groups at 4 hr after melatonin injection (Table 2). Again, the data represent the levels of melatonin found at 12:00 hr. In this case, however, animals were injected with melatonin at 08:00 hr and sacrificed 4 hr later.

To analyze the relationships between extracellular and intracellular melatonin levels, groups of control rats were injected i.p. with 0, 10, 40, 100, and 200 mg/kg bw melatonin at 08:00 hr and sacrificed 4 hr later. Fig. 7 shows the dose–response effects of melatonin injection in terms of the subcellular distribution in rat cerebral cortex. Cell membranes show a dose-dependent increase in melatonin concentration, reaching 10 times higher levels than in the cytosol. Moreover, whereas 10 mg/kg bw of melatonin significantly elevated the melatonin content in membranes ($P < 0.01$), cytosolic concentrations of melatonin did not change after the administration of 40 mg/kg bw melatonin ($P < 0.001$). Interestingly, mitochondria and nuclei show a saturation component: melatonin content in these compartments increased after the injection of 10 mg/kg bw melatonin ($P < 0.001$), but additional doses of the

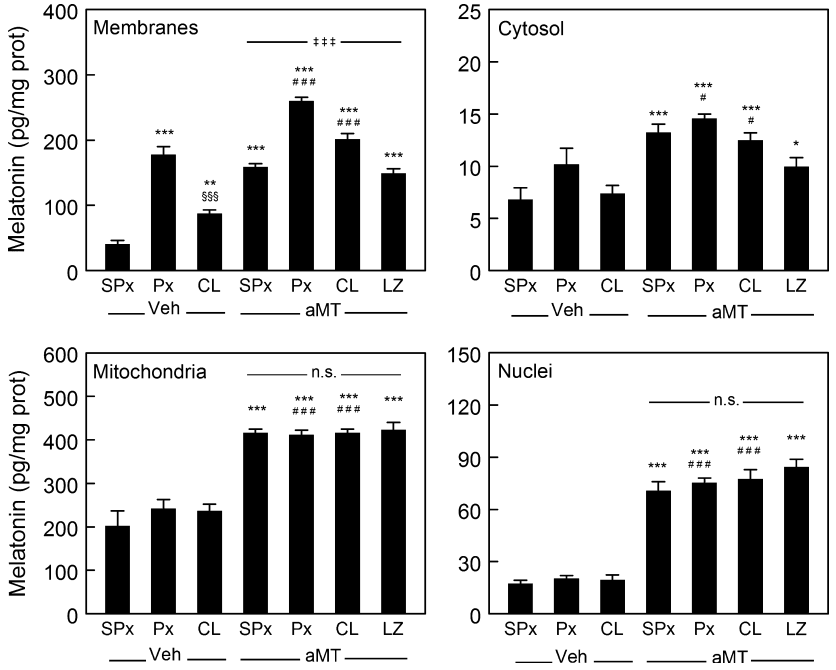

Fig. 5. Effects of Px, continuous light-exposed (CL), or luzindole (LZ) treatment on the cerebral cortical subcellular distribution of melatonin. Rats were sacrificed at 12:00 hr and injected with vehicle (sham-pinealectomized, SPx, Px, and CL), or melatonin (SPx, Px, CL, LZ) 2 hr earlier. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus SPx; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ versus vehicle groups; $^{\S\S\S}P < 0.001$ versus Px.

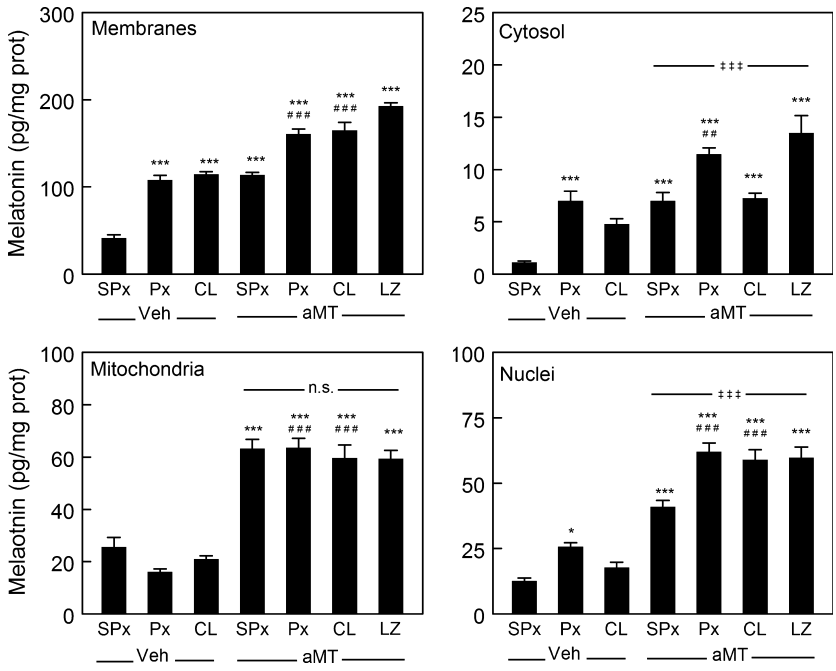

Fig. 6. Effects of Px, continuous light-exposed (CL), or luzindole (LZ) treatment on the liver subcellular distribution of melatonin (*N*-acetyl-5-methoxytryptamine). Rats were sacrificed at 12:00 hr and injected with vehicle (Veh) (sham-pinealectomized, SPx, Px, and CL), or melatonin (SPx, Px, CL, LZ) 2 hr earlier. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus SPx; $^{\#\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ versus vehicle groups.

indoleamine up to 200 mg/kg bw did not further increase the levels of melatonin in these cellular fractions. Similar pattern of behavior was found in liver (Fig. 8). Cellular membranes concentrate melatonin up to 10 times more than cytosol, whereas mitochondria and nuclei display comparable saturation mechanisms.

Discussion

The initial analysis of the results reveals new important aspects of melatonin physiology and pharmacology, which can be summarized as follows: (i) the existence of different subcellular distributions of melatonin; (ii) an absence of circadian fluctuations in the intracellular melatonin; (iii) the presence of a constitutive inhibition of intracellular melatonin production by pineal melatonin; (iv) the lack of effects of membrane melatonin receptors MT1/MT2 on subcellular distribution of the indoleamine; (v) a greater capacity of cellular membranes to concentrate melatonin, thereby presumably controlling its access to the cell; and (vi) the existence of additional specific regulatory mechanisms in mitochondria and nuclei preventing the full access

Table 2. Subcellular distribution of melatonin 4 hr after the injection of 10 mg/kg melatonin intraperitoneally

				Melatonin			
	SPx	Px	CL	SPx	Px	CL	LZ
Brain							
Membranes	40.84 ± 5.66	177.77 ± 12.42	87.60 ± 5.42	124.34 ± 3.27	193.85 ± 4.83	149.92 ± 5.97	118.27 ± 3.34
Cytosol	6.88 ± 1.11	10.17 ± 1.55	7.39 ± 0.77	9.09 ± 0.57	11.30 ± 0.40	8.78 ± 0.43	7.39 ± 0.28
Mitochondria	202 ± 34.8	242 ± 20.4	237 ± 15.3	311 ± 6.1	315 ± 4.3	328 ± 13.7	323 ± 7.7
Nuclei	17.42 ± 1.92	20.22 ± 1.74	19.55 ± 2.87	46.34 ± 2.38	59.31 ± 2.69	59.83 ± 3.98	57.24 ± 1.77
Liver							
Membranes	41.80 ± 3.81	107.99 ± 5.13	114.38 ± 3.10	75.62 ± 2.79	134.46 ± 4.08	129.07 ± 4.02	156.73 ± 3.46
Cytosol	1.15 ± 0.13	7.02 ± 0.91	4.80 ± 0.51	4.26 ± 0.20	9.90 ± 0.61	5.65 ± 0.31	6.36 ± 0.64
Mitochondria	17.55 ± 5.99	16.11 ± 1.07	20.39 ± 3.18	39.97 ± 1.73	49.32 ± 2.63	42.32 ± 2.59	43.21 ± 0.88
Nuclei	12.58 ± 1.21	25.67 ± 1.58	17.72 ± 2.04	24.96 ± 1.86	36.01 ± 1.84	38.68 ± 3.88	38.18 ± 2.36

SPx, sham-pinealectomy; Px, pinealectomy; CL, continuous light exposure; LZ, luzindole treatment.
Values are expressed as the mean ± S.E.M.

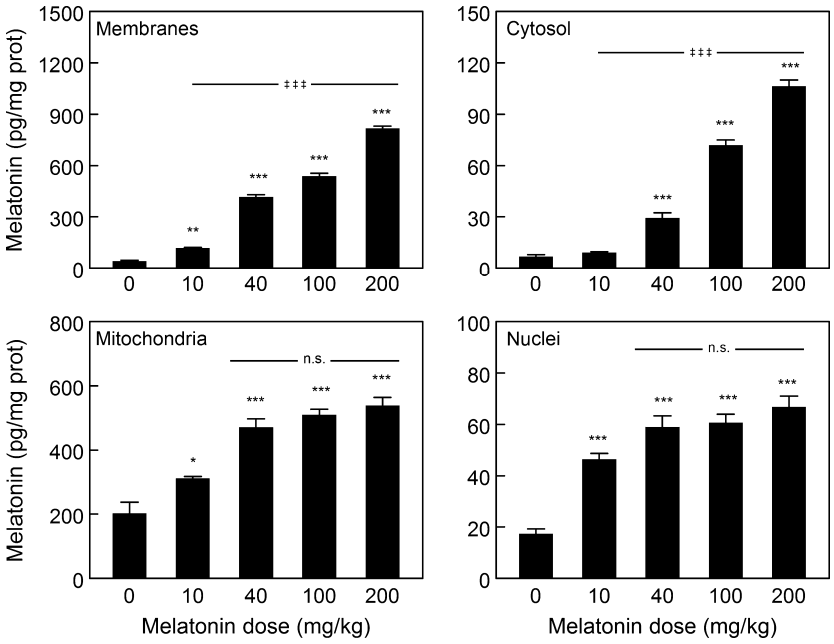

Fig. 7. Dose-dependent changes in the subcellular distribution of melatonin in rat cerebral cortex. Control rats were i.p. injected with either vehicle (0) or 10, 40, 100, and 200 mg/kg bw melatonin at 10:00 hr and sacrificed 2 hr later. $*P < 0.05$, $**P < 0.05$, and $***P < 0.001$ versus 0.

of melatonin to these organelles. Together, these data suggest that although biological membranes are permeable to melatonin, the indoleamine does not fully equilibrate within subcellular organelles, and specific mechanisms may regulate the amount of melatonin than can reach each subcellular compartment. The existence of these regulatory mechanisms reinforces the importance of melatonin in the biology of the cell, and it seems also to explain why high doses of melatonin are required to obtain sufficiently high intracellular levels for therapeutical purposes.

These results document the absence of circadian rhythms of extrapineal melatonin. Although there are fluctuations in melatonin concentrations over a 24-hr period in the different subcellular fractions, cosinor analysis indicated these rhythms were not circadian in nature. These data, together with the high concentration of intracellular versus extracellular melatonin, indicated that extrapineal melatonin functions different from the known pineal melatonin message [34]. These results are consistent with others that report the levels of this indoleamine in nuclei and mitochondria may be higher than in plasma [28, 35, 36]. In addition, we also show here that subcellular melatonin concentrations further increased after Px, suggesting that the extrapineal melatonin production, i.e., in liver and brain, is constitutively inhibited by circulating melatonin of pineal origin. Further support of this observation was the finding that CL rats, a treatment that inhibits the pineal melatonin production [37, 38], also increase the intracellular levels of melatonin. Analysis of the NAT and HIOMT activities and/or expression will be necessary to assess whether intracellular synthesis of melatonin varies with these manipulations.

The current subcellular distribution studies revealed significant differences among the different compartments studied. In brain cerebral cortex, melatonin levels were higher in mitochondria, followed by membranes, nuclei,

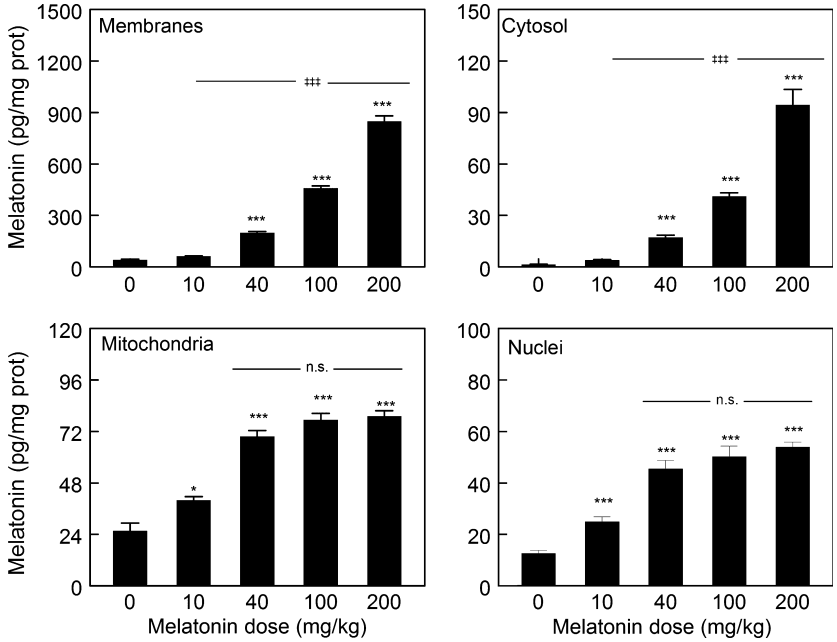

Fig. 8. Dose-dependent changes in the subcellular distribution of melatonin in rat liver. Control rats were i.p. injected with either vehicle (0) or 10, 40, 100, and 200 mg/kg bw melatonin at 10:00 hr and sacrificed 2 hr later. $*P < 0.05$, and $***P < 0.001$ versus 0.

and cytosol. These differences appeared also in Px animals, although in this case, the levels of melatonin increased mainly in cell membranes. In liver, maximal amounts of melatonin were identical in cell membranes, followed by mitochondria, nuclei, and cytosol. Px again increased melatonin in all subcellular structures. Similar observations have been made on the thymus, where the melatonin levels of SPx rats were lower than those in Px animals [39], although in this case, the subcellular distribution of melatonin was not determined.

The different distribution of melatonin may be related to specific functions in the cell. The brain has a high energy demand; thus, it uses 20% of total oxygen consumed, but it is only 2% of the body weight [40]. This feature reflects a high mitochondrial metabolic rate which in turn yields highly elevated reactive oxygen species (ROS) production [41–43]. Free radicals are especially dangerous in the brain because it has high concentrations of polyunsaturated fatty acids [44] and elevated levels of transition metals such as iron, which is involved in the generation of hydroxyl radicals [45]. These conditions, together with a low levels of cytosolic antioxidants [46, 47], make the brain excessively vulnerable to oxidative damage.

The presence of high concentrations of melatonin in brain mitochondria may reflect the homeostatic control of mitochondrial function by the indoleamine, improving their bioenergetic efficacy and reducing their production of ROS [16, 18, 41–44, 48, 49]. The elevated amount of melatonin may depend on a cellular adaptation to brain energy demand, allowing the use of large amount of oxygen; by contrast, the susceptibility of neurons and glia to oxidative damage is known to be prevented by melatonin [50–52].

In the case of liver, melatonin distributes homogeneously along the subcellular compartments, although mitochondrial content significantly increases at morning and evening, coinciding with the metabolic activity of the liver. These hepatic peaks of melatonin may be associated with the circadian control of the hepatic metabolic activity [53]. This hypothesis may be further supported by the observation that Px blunted these oscillations of melatonin.

As Px induces an accumulation of melatonin in cell membranes, we asked whether exogenous melatonin may also be accumulated in these structures. After the administration of 10 mg/kg bw, brain and liver cell membranes accumulated much of the circulating melatonin. Px, or CL exposure, both of which reduce significantly pineal melatonin production and thus, circulating melatonin [10], increased the amount of melatonin in these cell membranes in both vehicle- and melatonin-injected rats. Thus, it seems that the absence of circulating pineal melatonin modifies some feature of cell membrane enhancing its capacity to take up melatonin. Interestingly, LZ had little effect on these changes in melatonin, suggesting that MT1/MT2 membrane receptors of melatonin do not actively participate in the accumulation of melatonin by cell membranes. After melatonin administration, its concentration also increased in the cytosol, mitochondria, and nuclei, doubling the basal levels. These changes were generally independent of whether the animals were pinealectomized, subjected to continuous light, or treated with LZ. Thus, it seems that cell membranes act as a reservoir for melatonin, limiting the amount of melatonin available to the cell. The mechanism underlying these properties of the cell membrane remains unknown but probably may depend on specific membrane transporters.

We next asked whether the capacity of brain and liver cell membranes to retain melatonin could be saturated at high extracellular levels of the indoleamine, thus disrupting the homeostatic mechanism and allowing the free entry of melatonin into the cell. To address this question, rats were injected with different doses of melatonin 4 hr before their sacrifice. Our results show a dose-dependent accumulation of melatonin by the cell membranes, with no apparent saturation mechanism even at the highest dose used. At this

dose, i.e., 200 mg/kg bw, the amount of melatonin in cell membranes increased 20 times compared with their basal levels. Under these conditions, however, melatonin levels in mitochondria and nuclei increased only threefold, whereas in cytosol, melatonin concentration increased 15-fold. Hence, cytosolic concentrations of melatonin reached a final level 10 times lower than in cell membranes. These kinetic changes in cellular melatonin content were similar in both cerebral cortex and liver tissues. The interaction of melatonin with cell membranes has been evaluated, and it was suggested that it could easily scavenge both aqueous and lipophilic radicals [54]. The accumulation of melatonin in cell membranes reported in our data further supports this activity of the indoleamine, giving an in situ protection against free radical attack.

Another interesting observation was that at the dose of 40 mg/kg bw, the concentration of melatonin in mitochondria and nuclei of cerebral cortex and liver reached their maximal value. These findings were surprising because, to our knowledge, the amphiphilic properties of melatonin allowed it to cross all cell barriers, equilibrating on both sides [9]. In the light of the current findings, however, this point of view must be modified. The results suggest the existence of some regulatory mechanisms not only in the cell membrane, but also in the mitochondria and nuclei, which are able to modulate the transfer of melatonin from the membrane into the cell and from the cytosol to the nucleus and mitochondria. By regulating the intracellular concentration of melatonin, the cell membrane may act as a reservoir of melatonin, ready to be used by the cell whenever needed. Moreover, considering the intracellular effects of melatonin, mainly at nuclear and mitochondrial level, our results suggest that the therapeutical range of melatonin should be between 10 and 40 mg/kg bw in rats. To obtain similar therapeutical approach in humans, we can extrapolate these data to the human equivalent dose (HED) following the Reagan-Shaw et al. [55] conversion. This means that the human dose would oscillate between 1.6 and 6.5 mg/kg bw, i.e., 112 and 455 mg for an adult of 70 kg bw. Anyway, these doses in humans require further evaluation.

We next asked whether there exists a regulatory mechanism to control melatonin distribution into the cell. A response may come from the antioxidant and anti-inflammatory properties of melatonin. Melatonin is a major intracellular antioxidant, and this property is reflected, among other considerations, by its ability to maintain the glutathione (GSH) homeostasis by acting on several pathways: increasing GSH synthesis, augmenting the expression and activity of the γ-glutamylcysteine synthase [56], recovering GSH from oxidized glutathione (GSSG) through the induction of expression and activity of GSH reductase [16, 57, 58], and reducing the GSH consumption owing to the potent free radical scavenger ability of melatonin, thus decreasing the oxidative stress status [15, 41–43, 59–65]. GSH is necessary for a series of reactions in the cell leading to a defense against free radicals and detoxifying xenobiotics via GSH S-transferases. Additionally, GSH is also present in the cell nucleus, where it has been traditionally related to antioxidant protection of the genome [66]. Moreover, there is increasing evidence suggesting a role of GSH in nuclear homeostasis and cell proliferation, and nuclear GSH depletion prior to irradiation causes DNA fragmentation and apoptosis [67]. GSH moves to the nucleus when the cell is ready to proliferate, regulating a series of events necessary for cell division [68]. Thus, high GSH levels in the nucleus are directly related to cell proliferation. In preventing a GSH-dependent hyper-reduced status in the nucleus, cell and nuclear membranes control the intracellular amount of melatonin. This mechanism should now be considered regarding the oncostatic properties of melatonin. The observation may be generalized to other cell compartments. In fact, if melatonin distributes homogeneously in all subcellular compartments, the intracellular redox status of the cell will move to a high hyper-reduced status, altering many redox reactions.

The data of this study reveal that melatonin physiology is more complex than previously supposed. Melatonin concentrations vary over a 24-hr period in distinct subcellular compartments, and these changes probably indicate that melatonin may regulate separately the redox status in each compartment. Thus, melatonin becomes a much more important intracellular antioxidant, because it can selectively change in those subcellular structures where it is required. Among other considerations, the anti- and proapoptotic actions of melatonin in normal and cancer cells, respectively, might be a consequence of the alteration in the regulatory mechanisms affecting subcellular melatonin distribution in the latter, allowing the free influx of melatonin into the cell, exerting its oncostatic and proapoptotic effects. Also, importantly from a therapeutic point of view, is the fact that our data support the use of high doses of melatonin, as they seem to be necessary to reach subcellular concentrations sufficient to exert pharmacological effects. Finally, the existence of mechanisms controlling subcellular melatonin distribution may also explain the low toxicity of the indoleamine when it is used even at high doses. It is clearly of major interest to gain information regarding the mechanism(s) involved in the cellular control of melatonin distribution.

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