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Influence of Circadian Time and Lighting Conditions on Expression of Melatonin Receptors 1 and 2 in Murine Lymphocytes

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Abstract. *Aim: To investigate the expression of membrane melatonin receptors in murine thymocytes, CD4$^+$ T-cells, bone marrow cells and B-cells according to melatonin concentration in the blood. Materials and Methods: The levels of mRNA of melatonin receptors were investigated in the cells isolated during the day or during the night, corresponding to low and high melatonin concentrations in the blood. Results: Low levels of Mtnr1 and Mtnr2 transcripts were detected in thymocytes and splenic B-cells. The expression of membranous melatonin receptors in B-cells corresponds to melatonin concentration in the blood. Conclusion: The expression of Mtnr1 and Mtnr2 in murine lymphocytes is very weak. Melatonin may be involved in regulation of the expression of Mtnr1 and Mtnr2 in murine B-cells.*

The main role of melatonin is the regulation of circadian and seasonal biorhythms, however, it influences many other functions, including immune response. Melatonin is mainly produced by the pineal gland during the night in response to darkness and its production is inhibited by light (1). The secretion of melatonin reaches a maximum level at midnight and gradually decreases by the morning. Melatonin is also synthesized extrapineally in different tissues and cells,including the cells of the immune system (2).

Melatonin can bind three types of receptors: membranous receptors Mt1 and Mt2 (Mtnr1 and Mtnr2), cytosolic receptor Mt3 and nuclear receptors (3). The best characterized melatonin receptors, Mtnr1 and Mtnr2, belong to the G protein-coupled receptor superfamily (4). Mtnr1 and Mtnr2 are widely expressed by immune system cells. Mtnr1 and Mtnr2 were detected in the spleen and thymus (5-12). Mtnr1, additionally, was found in monocytes, B- and T-cells, thymocytes, and natural killer cells (6, 13). Melatonin acting *via* Mtnr1 increases interleukin-2 (IL2) production and proliferation in lymphocytes (2). Mtnr2 is involved in increased proliferation of murine splenocytes (11), and inhibition of rat leukocyte rolling (14).

Although Mtnr1 and Mtnr2 expression has been studied in other organisms, little is still known about their expression in organs and cells of the mouse immune system. Moreover, there are no data on the regulation of the expression of murine Mtrs. The amount of melatonin in the blood and the immunological response varies during the day and night (15, 16), but nothing is known about the influence of melatonin concentration on the expression of Mtnr1 and Mtnr2 in mice. Therefore, we investigated the expression of MTRs in murine lymphocytes (thymocytes, bone marrow cells, purified splenic B-cells, lymph node CD4$^+$ T-cells) isolated from mice kept under different lighting conditions.

Materials and Methods

Experimental animals. BALB/c mice were bred and housed in our animal facility. The experiments with animals were performed according to international ethical standards. The research protocol was submitted to and approved by a Lithuanian State Food and Veterinary Service (permission number 0225). The rodents were given *ad libitum* access to food and water and maintained under a 12/12-h light/dark cycle. One group of mice (LD) was kept under normal light/dark conditions; another group (LL) was kept under constant artificial lighting (around 50 lux) for about one week before experiments. In some cases, daily melatonin (Sigma-Aldrich, St.Louis, MO, USA) injections (5 mg/kg) were given for one week before the experiment: the LL night+mel group were given melatonin injections at the time corresponding to the beginning of the dark time for LD mice; the LD day+mel mice were injected at the beginning of the light time. The animals were sacrificed by cervical dislocation 4 h after beginning the light (LD day) or dark

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(LD night) time. The mice kept under constant lighting (LL day) were sacrificed at the same time as LD day mice.

Cell preparation. Thymocytes, splenocytes, lymph node cells and bone marrow cells were isolated from 8- to 9-week-old BALB/c mice. Splenic B220+ B-cells and lymph node CD4+ T-cells were isolated using phycoerythrin (PE)-labeled antibodies to B220 or CD4 and anti-R-PE Magnetic Particles-DM (BD Biosciences, San Jose, CA, USA). Cell isolation was performed according the manufacturer's recommendations.

RNA isolation and cDNA synthesis. RNA was extracted using GeneJET™ RNA Purification Kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) according the manufacturer's instructions. To remove DNA contamination, RNA was incubated for 30 minutes at 37˚C with DNase (final concentration 0.1 U/ml). cDNA was synthesized using Maxima Reverse Transcriptase and random hexamer primers (Thermo Fisher Scientific Baltics, Vilnius, Lithuania).

The polymerase chain reaction (PCR). PCR was performed in a Rotor-Gene RG 6000-time real-time PCR instrument. Three pairs of primers for Mtnr1 were used. Primers *Mtnr1* (A) were GGGCCCCACTCAACCTCATAG and AGCAGTAAGACCCCAA CCAGTGTG; primers *Mtnr1* (B) were ATCGCCATCATGCCC AACCT and TAACTAGCCACGAACAGCCACTCT; primers *Mtnr1* (C) were CCGCAACAAGAAGCTCAGGAACTC and TCGTACTTG AGGCTGTGGCAAATG. The primers for *Mtnr2* were AACCGCTA CTGCTGCATCTGTCAT and AAACTGCGCAAATCACTCGG TCTC, the primers for house keeping gene Hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) were GCTGGTGAAAAGG ACCTCT and CACAGGACTAGAACACCTGC. All primers [except *Mtnr1*(C) 1 were designed using Lasergene.v.7.1 software, DNASTAR, Madison, WI, USA. The sequences of *Mtnr1*(C) were as described by Carrillo-Vico *et al*. (7). The cDNA was amplified in a reaction containing 2 μl of cDNA, 2 μl of 10×PCR buffer, 2 μl of 2 mM dNTP/dUTP mix, 2 μl of 25 mM MgCl, 4 μl of betain, 1 μl of 10 pM of each 5'- and 3'-primer, 26.6 μM of a fluorescence dye SYTO9 (Invitrogen, Carlsbad, CA, USA), 4.5 μl of nuclease-free water, 0.4 units of Uracil-DNA glycosidase and 0.5-0.75 units of Hot start *Taq* polymerase (Thermo Fisher Scientific). The PCR reaction was started by initial 2-min PCR step at 50˚C and 5 min at 95˚C followed by 40-45 cycles of target cDNA amplification (45 s at 95˚C, 45 s depending on the primers from 54˚C to 61˚C and 45 s at 72˚C). The last elongation was 2 min for 72˚C. To exclude the contamination of genomic DNA, control PCRs were performed using isolated RNA (without cDNA synthesis). PCR products were separated by electrophoresis in 2% agarose gels, and visualized using an UV illuminator in the presence of ethidium bromide.

Detection of melatonin in mouse serum. The blood samples for melatonin detection were obtained from mice 4 h after beginning of the light or 4 h after beginning of the dark time. Melatonin in serum was detected using enzyme-linked immunosorbent assay kit (Cloud-clone Corp. Houston, TX, USA)

Results

Melatonin concentration in the serum of BALB/c mice. The melatonin concentration in the serum of LD and LL mice was determined. Blood was obtained at midday (4 h after beginning of the light time) and at midnight (4 h after beginning of the dark time). The melatonin concentration was increased in the sera of LD-night mice compare to LD-day and LL-day mice (Figure 1). The LL night mice had higher melatonin level than LL-day mice, but lower than LD-night mice.

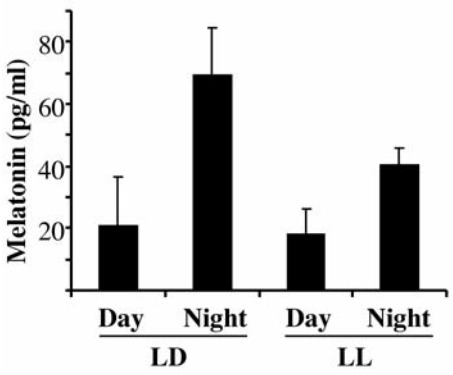

Figure 1. *The level of melatonin in the serum of BALB/c mice. The melatonin was measured in the serum of mice kept under normal light/dark (LD) or constant light (LL) conditions. Blood was obtained 4 h after beginning of the light (day) and 4 h after beginning of the dark (night) time. The average of melatonin concentration determined in two mice is shown.*

Expression of Mtnr1 and Mtnr2 in lymphocytes. The transcripts of *Mtnr1* and *Mtnr2* were analyzed using three different primer pairs for *Mtnr1* and one primer pair for *Mtnr2*. PCR was performed from several samples of mRNA, prepared from different mice (Table I). The PCR from every sample was carried out in duplicates or triplicates. The levels of mRNA of *Mtnr1* and *Mtnr2* were very low. PCR products had relatively high Ct values (Ct=33-40). We did not find transcripts of *Mtnr1* and *Mtnr2* in the cDNA from CD4+ T-cells and bone marrow cells (data not shown). mRNA of *Mtnr1* and *Mtnr2* was detected in thymocytes isolated from most LD day, LL night and LL night+mel mice. PCR was not positive in all replicates of the same sample, indicating that the concentration of transcripts is very low and ranges around the detection limit (at least one copy of cDNA present in one aliquot of template and absent in the second aliquot); such samples are marked +/- in Table I. PCR product was not obtained with all primer pairs.

Transcripts of *Mtnr1* and *Mtnr2* in B-cells were not detected in the samples from LD-day and LL-night mice, but were found in the samples prepared from LD-night mice. Melatonin injection into the LL mice (LL night+mel mice) induced the expression of Mtnr1 and Mtnr2.

In conclusion, the expression of *Mtnr1* and *Mtnr2* in lymphocytes is very weak or undetectable. The presence of *Mtnr1* and *Mtnr2* transcripts in splenic B-cells depends on lighting conditions.

Table I. *Expression of melatonin receptors Mtnr1 and Mtnr2 in thymocytes and B-cells isolated from spleen of mice kept under constant light (LL) and 12/12 light/dark (LD) conditions. Samples were taken 4 h after the beginning of the light (day) or 4 h after the beginning of the dark (night) time. The expression of melatonin receptors was analyzed by rtPCR.*

Receptor	Experiment no.	cDNA source	Primers	LD night	LD day	LL day	LD day+mel	LL night	LL night+mel
Thymocytes									
Mtnr1	I	1	Mtnr1 (A)	+	+	ND	ND	ND	ND
			Mtnr1 (B)	–	+	ND	ND	ND	ND
	II	2	Mtnr1 (A)	–	+	–	ND	ND	ND
			Mtnr1 (B)	+/–	+/–	–	ND	ND	ND
			Mtnr1 (C)	–	–	–	ND	ND	ND
	III	3	Mtnr1 (A)	–	–	–	ND	ND	ND
			Mtnr1 (B)	–	–	–	ND	ND	ND
			Mtnr1 (C)	–	+/–	–	ND	ND	ND
	IV	4	Mtnr1 (A)	+	–	ND	–	+/–	+/–
			Mtnr1 (B)	+	–	ND	–	+/–	+/–
			Mtnr1 (C)	–	+/–	ND	–	+/–	–
		5	Mtnr1 (A)	+/–	–	ND	–	+/–	+/–
			Mtnr1 (B)	+	–	ND	–	+/–	+/–
			Mtnr1 (C)	–	+	ND	–	+	–
		6	Mtnr1 (A)	–	–	ND	–	–	+
			Mtnr1 (B)	+	+/–	ND	–	+	+
			Mtnr1 (C)	–	–	ND	–	–	–
Mtnr2	I	1	Mtnr2	–	+	ND	ND	ND	ND
	II	2	Mtnr2	–	+	++	ND	ND	ND
	III	3	Mtnr2	–	–	–	ND	ND	ND
	IV	4	Mtnr2	+	–	ND	–	+/–	+
		5	Mtnr2	+	–	ND	–	–	+
B-Cells									
Mtnr1	I	1	Mtnr1 (A)	+/–	–	–	ND	ND	
			Mtnr1 (B)	+/–	–	+/–	ND	ND	
	II	2	Mtnr1 (A)	+/–	ND	ND	ND	ND	
			Mtnr1 (B)	–	ND	ND	ND	ND	
	III	3	Mtnr1 (A)	+/–	ND	ND	–	+/–	
			Mtnr1 (B)	+/–	ND	ND	–	+/–	
			Mtnr1 (C)	–	ND	ND	–	–	
		4	Mtnr1 (A)	+/–	ND	ND	–	+/–	
			Mtnr1 (B)	+/–	ND	ND	–	+/–	
			Mtnr1 (C)	–	ND	ND	–	–	
		5	Mtnr1 (A)	–	ND	ND	–	–	
			Mtnr1 (B)	–	ND	ND	–	+/–	
			Mtnr1 (C)	–	ND	ND	–	–	
Mtnr2	I	1	Mtnr2	+/–	–	+/–	ND	ND	
	II	2	Mtnr2	+	ND	ND	ND	ND	
	III	3	Mtnr2	+/–	ND	ND	–	+/–	
		4	Mtnr2	+/–	ND	ND	–	+	
		5	Mtnr2	+/–	ND	ND	–	+/–	

+ mel: Daily melatonin injections for one week before the experiment. LD day + mel –: Melatonin injected at the beginning of the light time. LL night + mel –: Melatonin injected at time corresponding to the beginning of the dark time for LD mice. +: PCR positive, –: PCR negative, +/–: some replicates from the same sample were positive and some were negative, ND: not done.

Discussion

Herein, we investigated the expression of Mtrs in thymocytes, bone marrow cells, $CD4^+$ T-cells, B-cells of BALB/c mice. Our results demonstrated that the levels of *Mtnr1* and *Mtnr2* transcripts in the tested cells were very low. We found expression of these receptors in the thymocytes and splenic B-cells, while such transcripts were not detected in $CD4^+$ T-cells and bone marrow cells. The distribution of *Mtnr1* and *Mtnr2* mRNA was similar (they were found together in the same cells). It is interesting that melatonin surface receptors can form Mtnr1–Mtnr2

heterodimers (17), which also shows that both receptors are expressed together. Mtnr1 and Mtnr2 are expressed in thymus and spleen of rats (6, 12), tropical rodent *Funambulus pennant* (8, 9), the avian *Perdicula asiatica* (5), and the chicken (10). In addition, the expression of Mtnr1 and Mtnr2 in thymus and spleen of Swiss mouse was investigated by Carrillo-Vico *et al*. (7). The authors found both Mtnr1 and Mtnr2 transcripts in the thymus, however, only Mtnr1 transcripts were detected in the spleen. These receptors were analyzed by PCR followed by Southern blot but PCR product was not detectable on the agarose gel, which demonstrates the low expression of the investigated receptors. Mtnr2 was absent in the murine spleen (7). A weak signal of *Mtnr1* mRNA was also obtained in human PBMC, monocytes, T- and B-cells, and natural killer cells (13). Taken together, these data show that the expression of Mtnr1 and Mtnr2 in lymphocytes is very low.

The amount of melatonin drops during aging, but the levels of Mtrs in thymus were significantly higher in 12-month-old rats than in 12-week-old ones (12). Comparing Mtnr1 expression in rats of the same age, Jimenez-Jorge *et al*. detected more *Mtnr1* transcripts in the thymus at day time than at night time (18). Such results argue that the levels of surface Mtrs depend on the concentration of melatonin in the blood. These results show that melatonin negatively regulates the transcription of its own receptors in thymus. Hence we expected to obtain similar results in mice. However, we did not find a link between Mtnr1/Mtnr2 expression and melatonin concentration in the blood or circadian time. One possible explanation is that the amounts of *Mtnr1* and *Mtnr2* transcripts in the murine thymus were very low and varied between individual animals.

We detected mRNA of *Mtnr1* and *Mtnr2* in splenic B-cells only in the night (LD-night mice), which corresponds to the increased melatonin concentration in the blood. Age-dependent decrease of melatonin concentration in the serum and decrease in Mtnr1 and Mtnr2 expression in spleen was observed in rats (12) and *F. pennanti* (9). The circulating melatonin concentration and Mtr expression in thymus and spleen of *F. pennanti* were decreased during the long days of summer and increased during the short days of winter (8). Therefore, the expression of Mtrs depends on the concentration of the melatonin in the blood, which depens on the circadian time, length of day and animal age.

Ahmad *et al*. showed that melatonin directly regulates the expression of its membranous receptors. Physiological doses of melatonin up-regulated Mtnr1 and Mtnr2 expression in *F. pennanti*, while higher doses down-regulated the expression of both receptors in splenic cells (*in vivo* and *in vitro*) (19). Our experiments showed also that melatonin up-regulated the expression of its receptors. We did not find mRNA of *Mtnr1* and *Mtnr2* in B-cells when the melatonin concentration in the blood was supposedly low (LD day and LL night mice). Daily melatonin injections administered to mice kept at constant light (LL night+mel mice) restored the expression of Mtnr1 and Mtnr2. Differently from rodents, membrane Mtrs are down-regulated in the spleen of *P. asiatica* (5) but up-regulated in the lungs by melatonin during the night (20). Melatonin regulates the expression of Mtnr1 and Mtnr2 not only dependent on the concentration of the melatonin and circadian time, but also on the biological species and origin of the cells.

The increased Mtnr2 expression in B-cells during the night could be important for priming of immune response. We previously showed that melatonin modulates antibody secretion in mice *via* Mtnr2. The titter of specific antibody in the blood was higher when immunizations (B-cell priming) were carried out in the night compared to those in the morning (15), corresponding to higher melatonin concentration in the blood and increased Mtnr2 in B-cells. These data suggest that melatonin positively-regulates B-cell priming and antibody production.

In conclusion, melatonin may be involved in the regulation of the expression of Mtnr1 and Mtnr2 in murine B-cells.

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