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Regulation of MT1 and MT2 Receptors in Pineal Gland with Light/Dark Cycle: A Novel Approach

[Aydınlık/Karanlık Döngüsü ile Pineal Bezde MT1 ve MT2 Reseptörlerinin Regülasyonu: Yeni Bir Yaklaşım]

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ABSTRACT

Aim: Melatonin is an endogenous hormon, which oscillates from pineal gland and affects through melatonin receptors (MT1 and MT2) denoting different physiologic roles, accountable to regulate biological rhythm. This study is to known first study to show the regulation of melatonin synthesis by the melatonin receptor expressions in pineal gland.
Materials-Methods: 200-250 gr weighted, 50 male Sprague Dawley rats were being examined with 5 different (n=10, 12/12hr light/dark (L/D), 24/0hr, 0/24hr, 8/16 hr L/D, 16/8 hr L/D) cycles for one week. Plasma melatonin levels were measured with ELISA kits and pineal gland receptors' level with "Real Time Polymerase Chain-Reaction (Real-Time PCR)". For the statistical analysis; reallocation randomization test, One-way Anova, Mann-U tests were applied.
Results: Plasma melatonin levels in 0/24hr L/D group were examined statistically higher than control group and 24/0 hr L/D loops applied group ($p<0.05$). In paralel with MT1 ($p<0.001$) and MT2 ($p<0.005$) receptor expressions were semanticly upregulated at dark but downregulated in light.
Conclusion: As similar with pineal gland receptors, melatonin levels increased highest rank of 26 pg/ml in dark, the lowest rank of 10 pg/ml in light exposure, reflecting in pineal gland Gi-dependent melatonin receptor expressions detained melatonin level in a particular rank. This study revealing firstly that instead of signals of SCN, MT1 and MT2 receptor expressions may regulate the plasma melatonin levels and the circadian clock.
Keywords: Melatonin, MT1, MT2, pineal gland

ÖZET

Amaç: Melatonin, pineal bezden salınan ve melatonin reseptörleri (MT1 ve MT2) ile çeşitli fizyolojik etkiler gösteren, biyolojik ritmin düzenlenmesinden sorumlu endojen bir hormondur. Bu çalışma melatonin sentezinin pineal bez melatonin reseptörleri tarafından kontrol edilebileceğini gösteren ilk çalışmadır.
Materyal-Metod: Çalışmada 200-250 gr, 50 adet, erkek, Sprague Dawley sıçan özel ritim kafeslerinde bir hafta süre ile 5 farklı (n=10, 12/12, 0/24 s aydınlık/karanlık (A/K), 8/16 s A/K, 16/8 s A/K, 24/0 s A/K) sirkadiyen ritme tabi tutulmuş ve plazma melatonin düzeyleri ELISA kiti ile, pineal bez reseptör düzeyleri ise "Gerçek Zamanlı Polimeraz Zincir Reaksiyonu (Real-Time PCR)" ile ölçülmüştür. Elde edilen verilerin istatistiksel analizinde reallokasyon randomizasyon testi, One-way ANOVA, Mann-Whitney-U testleri kullanılmıştır.
Bulgular: Çalışmada plazma melatonin düzeyleri 0/24s A/K grubunda, kontrol grubuna ve 24/0 s A/K döngüsü uygulanan guruba göre istatistiksel olarak yüksek bulunmuştur ($p<0.05$). Buna paralel olarak MT1 ($p<0.001$) ve MT2 ($p<0.05$) reseptör ekspresyonları ise karanlıkta anlamlı olarak artarken aydınlıkta ise azalmıştır.
Sonuç: Çalışmada, pineal bez melatonin reseptörleri gibi sürekli karanlık uygulanan grupta melatonin düzeylerindeki artış belirli bir düzeyin üstüne çıkmazken (26 pg/ml), sürekli aydınlık uygulanan grupta da yine belirli bir düzeyin (10 pg/ml) altına düşmemiştir. Bu nedenle de pineal bezde Gi aracılı melatonin reseptör ekspresyonunun plazma melatonin düzeylerini belirli bir düzeyde tutmayı sağladığı düşünülmektedir. Plazma melatonin düzeylerinin SCN dışında hormonun salım yeri olan pineal bezdeki reseptörlerin ekspresyonları ile düzenlendiğinin ilk kez gösterildiği bu çalışma oldukça önem taşımaktadır.
Anahtar Kelimeler: Melatonin, MT1, MT2, pineal bez

Introduction

Melatonin (N-acetyl-5 methoxytryptamine) was characterized after its isolation from bovine pineal tissue by Lerner et al. [1,2] roughly 50 years ago; this indole is known the major secretory product of the pineal gland which thereby regulates the circadian rhythm in all mammals. In addition to this regulation, the intervening years since its structural identification it has been functionally linked to retinal physiology [3], tumor inhibition [4], free radical scavenging and antioxidant effects [5].

The major regulator of melatonin production is the prevailing light/dark environment, and this is accomplished by a series of neurons that originate in the retinas and eventually end in the pineal gland [6].

The circuitous pathway between the suprachiasmatic nucleus and the pineal gland conveys information about the light/dark environment to the pineal gland and thereby determines the melatonin synthesis cycle [6].

The regulation of melatonin production in the pineal gland has been defined in significant detail. The primary neurotransmitter released from the postganglionic symphathetic terminals that terminate in the pineal gland is norepinephrine (NE, noradrenalin); during darkness at night, NE is discharged onto the pinealocytes, the endocrine cells of the gland, where it couples especially to beta adrenergic receptors. These receptors are a kind of Gs receptors which leads to a marked rise in intracellular cAMP levels, to de novo protein synthesis and eventually to the stimulation of the rate-limiting enzyme in melatonin production, arylalkylamine-N-acetyltransferase (AA-NAT) [6].

Once synthesized, melatonin quickly diffuses out of the pinealocytes into the rich capillary bed within the gland [7, 8]. As result the blood levels rise at night and the concentration of melatonin in these fluids is generally accepted as an index of its concurrent synthesis within the pineal gland. Circulating nocturnal levels of melatonin are commonly 10-20 times higher than daytime concentrations [9].

Melatonin has a variety of means by which it influences the physiology of the organism; some of these actions are receptor mediated (e.g. classical endocrine effects) while others are receptor-independent (e.g. free radical scavenging). The distribution of the two known melatonin receptor MT1 and MT2 is remarkably widespread in mammalian tissues like SCN [10], gastrointestinal tract, hypothalamus and retina [11]. Membrane melatonin receptors have seven transmembrane domains and they are in the superfamily of G-protein-coupled receptors. These receptors of melatonin are found to be a Gi type of G proteins which inhibits the cAMP levels in the action sites [9].

Up to date melatonin was known to act at the level of the SCN to modulate its activity and influence circadian rhythms via its receptors on SCN. Therefore in this study we identified the MT1 and MT2 receptors in pineal gland by Real-Time PCR and we tried to show the correlations between plasma melatonin levels and pineal gland receptor expressions under different light/dark cycles where we would be able to identify if the plasma melatonin levels has an autoregulation mechanism on the pineal gland directly effecting its secretion.

Materials and Methods

The study had been performed in Erciyes University, Medical Faculty, Physiology Department and Balearic Islands University, Biology Faculty, Molecular Biology and Biochemistry Department.

Materials

Oligonucleotide primer sequences for MT1 and MT2 receptors, LightCycler-FastStart DNA Master SYBR Green I for real-time PCR and (Roche; Light Cycler) (Real-Time PCR) Tripure isolation reagent was purchased from Roche Diagnostics (Basel, Switzerland). RT-PCR chemicals were from Applied Biosystems (CA, USA). Routine chemicals used were supplied by Pronadisa (Madrid, Spain), Panreac (Barcelona, Spain), Sigma-Aldrich and Roche Diagnostics.

Animals and experimental protocols

Animal experiments were performed in accordance to general guidelines approved by our institutional ethics committee and were performed with 50 Swiss Albino male rats, weighing in average 200-250 gr that were supplied from Erciyes University Experimental Clinical Research Center. Through the experimental procedures they were fed with standard pellet food and tap water in standard room temperature (20-24°C) and humidity (%70-75). The rats were separated to 5 different groups in 10 and were subjected to the cycles in specially-designed cages under different light-dark cycles for one week time. The light cycle applied to the subjects was 12/12 hr L/D, 0/24 hr L/D, 8/16 hr L/D, 16/8 hr L/D, 24/0 hr L/D. The experiments followed a protocol approved by the local animal Ethics Committee.

Sample collection

At the end of one week all the rats in the groups were sacrificed at 10:00 in the light or dark period which was necessary for the cycle for that group after ketamine anesthesia (40 mg/kg). After the whole blood received from abdominal aorta via heparinized injector, the skull was opened and the pineal glands were removed immediately and placed into the serum physiologic solution which involves RNAase enzyme inhibitor. The plasma samples and the pineal glands were stored at -80ºC until used for total RNA isolation and plasma melatonin measurements.

Plasma melatonin levels

The level of plasma melatonin is measured by Melatonin

ELISA kit (IBL, Cat No: RE54021 Turkey). The assay procedure follows the basic principle of competitive ELISA whereby there is competition between a biotinylated and a non-biotinylated antigen for a fixed number of antibody binding sites. The amount of biotinylated antigen bound to the antibody is inversely proportional to the analyte concentration of the sample. When the system is in equilibrium, the free biotinylated antigen is removed by a washing step and the antibody bound biotinylated antigen is determined by use of anti-biotin alkaline phosphatase as marker and p-nitrophenyl phosphate as substrate. Quantification of unknowns is achieved by comparing the enzymatic activity of unknowns with a response curve prepared by using known standards and expressed with pg/ml. The analytical sensitivity of the kit was 1.6 pg/ml (our results were with a precision of intra-assay %CV:%5.6, inter-assay %CV:%15.2)

Analysis of MT1 and MT2 expressions by Real-Time PCR

Total RNA was extracted from the samples by using TriPure Isolation Reagent (Roche, Mannheim, Germany) according to the manufacturer's protocol. After cell lysis and RNA extraction, in attempt to discard DNA contamination, before cDNA synthesis, RNA samples were undergoing a purification process by using DNAse I (Roche, 2U/10 µg RNA). The quantity and quality of the RNA was assessed spectrophotometrically at 260 m and 260/280 nm, respectively. 1microgram of the total RNA was reverse transcribed using PCR (Thermal Cycler, denaturation; 90ºC in one minute, reverse transcription; 42°C for 60 minutes and 95ºC for 5 minutes). In preliminary studies, SYBR Green-labeled PCR products were evaluated by agarose gel electrophoresis. cDNA samples were diluted 1/10, and aliquots were frozen at -80ºC until the PCRs were carried out.

Real-time PCR was performed using SYBR Green detection technology in a LightCycler Rapid Thermal Cycler (Roche Diagnostics). Each reaction contained 1 µl of Lightcycler-FastStart DNA Master SYBR Green I (containing FastStart Taq DNA polymerase, dNTP mix, reaction buffer, $MgCl_2$ and SYBR Green I dye; Roche Diagnostics), 0.5 µmol/L of each specific primer, 2 mmol/L of $MgCl_2$ and 3 µl of the cDNA dilution in 10 µl of the total reaction volume.

Oligonucleotide primer sequences used for real-time PCR were forward, 5 - ACTGGAAGGCCAATACAGTTGA, reverse primer base sequence 5 - ATGTTCGCAGTGTTTGTGGTTT for MT1, forward 5 -GGCGGGGAGGAAATAAGA, reverse primer base sequence: 5 -GTTGCGATACACAGACAGGA for MT2, and forward primer 5'-GAG GTG AAA TTC TTG GAC CGG-3'; reverse primer 5'-CGA ACC TCC GAC TTT CGT TCT-3' for 18S ribosomal RNA (18S) and forward primer 5'- GTG GTG TGT GAA GCT GTA GCC-3', reverse primer 5'- ACG GGC AAT GTG ATG GAC TC-3' for β-Actin which were used as housekeeping. For each oligonucleotide primer sequence, a basic local alignment search tool (NCBI, BLAST) revealed that an optimum sequence homology was obtained for the target gene.

In real time PCR below protocol was applied to cDNA getting from mRNA which belongs to MT1, MT2 receptors and 18S, and β-Actin housekeeping gene.

The amplification programs were as follows: a preincubation step for denaturation of template cDNA at 95ºC for 600 seconds for MT1 and MT2, followed by 32 cycles consisting of a denaturation step (95ºC 10 seconds for MT1and MT2 and 95ºC for 5 s for 18S), an annealing step (58ºC 10 seconds for MT1, 68ºC 10 seconds for MT2 and 60ºC for 8 s for 18S) and, finally, an extension step (72ºC 7 seconds for MT1 and MT2). Melting programs were 95ºC 5 second, 65ºC 180 second, 99ºC 1 second for MT1 and MT2 95ºC for 5 s, 65ºC for 15 s and 99ºC for 0 s. Cooling time was 95ºC for 1 second,40ºC for 20 second for MT1 and MT2.

Real-time PCR efficiencies were calculated on average of efficiencies from each sample, which were calculated by using the following formula: $e = (F/F0)^{1/(n-n0),}$ where F and F0 mean fluorescence values belonging to the linear segment of each PCR quantification curve and n and n0 represent their corresponding crossing points. MT1 and MT2 real-time PCR efficiencies were 1.89 and 1.87, respectively. PCR product sizes were 122 bp for MT1, and 64 bp for MT2. Serial dilutions of known quantities of recombinant plasmid DNA containing the the specific target sequences were used as standards in the PCR reactions, and the regression lines generated from "crossing point" values of standards were used to calculate the mRNA abundance.

In "Light Cycler" system; melting point and cDNA synthesis quantitative analysis were also performed with the software. Performing the synthesis values were identified beforehand. The quantification curve's are derived from standart curve's through "Second derive's maximum" method. This technique automatically calculates the cycle's number in which the fluorescent appears maximal. This point is also where the fluorescent is the fastest.

Statistical analysis

All data are expressed as the mean values of ten animals per group. Statistical analyses were performed by using a statistical software package (SPSS 13.0 for Windows, SPSS, Chicago, IL, USA). Differences between experimental groups in biochemical parameters were assessed by two-way analysis of variance (ANOVA) followed by Student's t test, as post hoc comparison. 0.05 was considered statistically significant.

The statistical PCR data analysis was performed by using the Relative Expression Software Tool. Differences in mRNA levels between groups were analyzed with the Pairwise Fixed Reallocation Randomization Test, a proper model to avoid the normal distribution of data.

Results

Plasma melatonin levels

Plasma melatonin levels have been ascertained statistically high in the group of in 0/24hr L/D applied group ($p<0.001$). In the other groups: the more the light the less the melatonin leves were found as expected and in the group of 24/0 L/D group the level of plasma melatonin stated almost the same as the control group (Figure 1).

MT1 and MT2 receptor expressions

Quantitative real-time PCR assays were established for 18S, β-Actin, MT1 and MT2. For each receptor run, a negative control was systematically added to, where in RNase-free water replaced complementer DNA. In addition a single peak was observed for each of the products by melting curve analysis performed routinely for all samples after amplification. No product was detected in the absence of RT in any assay for any of the products indicating that there was no genomic contamination.

As shown in Figure 2, MT1 receptor mRNA expression are expressed as the fold changes versus control group (12/12h L/D). The MT1 receptor expression was down regulated in the 24/0 (not significant) and 16/8h L/D groups ($p<0.05$), whereas it upregulated significantly in the 8/16h and 0/24h L/D groups ($p<0.001$).

The fold-changes of MT1 mRNA receptor expressions versus control group and the signifcance of the alterations are given in Table 1.

MT2 receptor mRNA expression are expressed as the fold changes versus control group (12/12h L/D). The MT1 receptor expression was down regulated in the 24/0 (not significant) and 16/8h L/D groups ($p<0.05$), whereas it upregulated significantly in the 8/16 and 0/24h L/D groups ($p<0.05$) (Figure 3).

The fold-changes of MT1 mRNA receptor expressions versus control group and the signifcance of the alterations are given in Table 2.

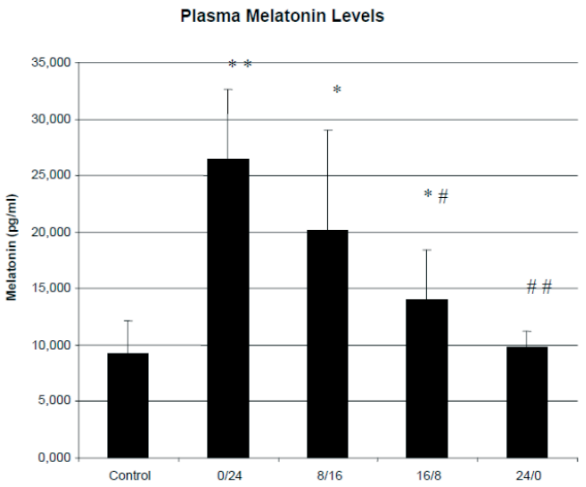

Figure 1. Plasma melatonun levels. Control: 12/12h L/D, *: Compared to control $p<0.05$, **: Compared to control $p<0.001$. #: Compared to 0/24h L/D $p<0.05$, # #: Compared to 0/24h L/D group $p<0.001$.

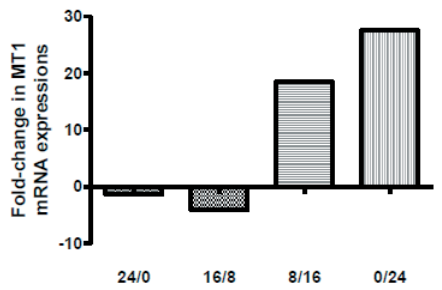

Figure 2. Real-Time PCR analysis of melatonin receptor MT1 mRNAin pineal gland. The results are expressed in fold changes versus control group (12/12h L/D). Significance of the data is given in Table 1. *: $p<0.05$.

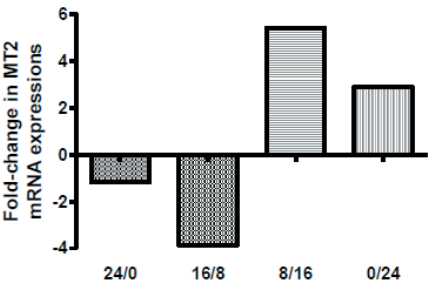

Figure 3. Real-Time PCR analysis of melatonin receptor MT2 mRNAin pineal gland. The results are expressud in fold changes versus control group (12/12h L/D). Significance of the data is given in Table 2. *: $p<0.05$.

Discussion

In mammals specific actions of melatonin are mediated by two different subtypes of Gi-coupled receptors which therefore decreases the cAMP levels in the action site [3, 12]. However, we know that in the pineal membrane the melatonin secretion is mediated by Gs-coupled beta-adrenergic receptors where the cAMP increase leads to melatonin secretion [6, 9]. Taking into this two well-known data, this study is designed to identify if there are MT1 and MT2 receptors on the pinealocyte membrane and if there are, how do they have an influence on the melatonin levels under different light/dark cycle alterations.

MT1 and MT2 receptors have been identified in several tissues like immune system, kidney, liver, heart, thymus and spleen [13] and also in hypothalamus, retina [14], Harderian gland and liver [11] and especially mainly in the SCN [10] in several articles. There are some factors that compromises the quantity of the melatonin the human pineal gland produces such as genetics, age and periods of the light/dark cycle. According to the traditional knowledge, light at night prevents the SCN from signaling the pineal gland to activate the molecular machinery to produce the melatonin. The use of artificial light (sometimes referred to as the misuse of the light) truncates the period of melatonin synthesis to an interval

Table 1. Up-regulation and down-regulation fold-changes of MT1 mRNA expressions

Groups	Up-regulation	Down-regulation	Fold-change	p values
Control vs 24/0h L/D		X	1.33	p>0.05
Control vs 0/24h L/D	X		27.509	p<0.001
Control vs 16/8h L/D		X	4.108	p<0.05
Control vs 8/16h L/D	X		18.454	p<0.001
24/0h vs 0/24h L/D	X		30.141	p<0.001

Table 2. Up-regulation and down-regulation fold-changes of MT2 mRNA expressions

Groups	Up-regulation	Down-regulation	Fold-change	p values
Control vs 24/0h L/D		X	1.17	p>0.05
Control vs 0/24h L/D	X		2.88	p<0.05
Control vs 16/8h L/D		X	3.87	p<0.05
Control vs 8/16h L/D	X		5.41	p<0.05
24/0h vs 0/24h L/D	X		3.66	p<0.05

shorter than it would normally be, thereby limiting the total amount of melatonin produced [15]. Light exposure has two basic functions on the melatonin synthesis cycle: acute light exposure at night (even if very short durations) inhibits melatonin production while alternating periods of light (and darkness) serve to synchronize the melatonin rhythm to 24h. Up to date SCN is the known circadian pacemaker where melatonin acts to modulate its activity. However, this study reveals for the first time the evidence of MT1 and MT2 receptors in the pineal gland itself which probably have an autoregulatory function to melatonin synthesis.

Plasma melatonin levels in human subjects [16] and melatonin receptor expressions in pleuronectiform species [17] in different light/dark conditions have been shown in some recent data. 10:00 am has been selected as a time for all the measurements including the plasma melatonin levels since in this experimental design our purpose was to determine the effects of light and dark alterations which reflects the exogenous impacts on the MT1 and MT2 expressions therefore 10:00 am is used to eliminate the endogenous circadian expression of melatonin. Endogenous melatonin starts increasing by the time of 8:00 pm and makes a peak at around 4:00 am and starts decreasing to the minimum levels at around 8:00 am. So that our study was designed to see the effects of exogenous regulation of melatonin with light and dark.

Commonly melatonin receptors have been visualized using radioactive 2- [125I]- iodomelatonin, and melatonin receptor mRNA levels have been quantified by Northern blot analysis. A more sensitive method reverse-transcriptase PCR reaction is also used in several tissues and SCN. Then Sallinen et al. [11], has quantified the melatonin receptor expressions in several tissues by Real-Time PCR which is a more sensitive detection method rather than the other methods. Also a novel study of Sanchez-Hidalgo [13] has also used this method to quantify the receptor mRNA levels in several tissues related to immune system. In our study, the Real-Time PCR technology is used for the first time in detection of MT1 and MT2 receptors in pineal gland. Not only we showed the expressions of these receptors in pinealocyte membrane and may have a modulatory function on its synthesis probably by the interactions of Gi and Gs receptor activation but also we used a sensitive quantitative method like Real-Time PCR for the detection of melatonin receptors on the pinealocyte membrane.

Although the MT1 and MT2 receptor expressions are expected as like the other action sites to be upregulated in light and down regulated in dark according to the melatonin quantity like all the other hormones and hormone-like substances do on their receptors in action sites, our results reveal that both of the receptors MT1 (much more than the MT2 probably due to the receptor affinity) and MT2 are down regulated during light and downregulated during the light period in pineal gland. And these levels were correlated with the plasma melatonin levels ($p<0.05$) both in dark and light. Our findings support the pancreatic MT-1 receptor mRNA upregulation during the dark period parallels the nocturnal rise in melatonin levels [18] but in contrast to the situation in SCN, as has been reported by Neu and Niles [19]. It also remains an open question whether the incidence of a generally exception of augmented MT1-receptor mRNA level in light group can be viewed as a compensatory effect in order to counteract reduced melatonin levels. A widely accepted concept assumes an inverse relationship between MT1-receptor transcription and plasma hormone concentration because of an inhibitory effect of melatonin on its own receptor [20]. However on the pineal gland this reverse hypothesis can be explained by the activation of Gs receptors which should be furt-

her increase the cAMP and leads to augmented plasma melatonin levels at dark and than the secreted melatonin thereby reaches to pinealocyte membrane effecting its own upregulated Gi-coupled receptors which would lead to decreased cAMP levels and would probably limit the exaggerated melatonin levels since this action on Gi receptors would be able to limit the melatonin secretion which is initiated with increased cAMP levels. Our hypothesis can be confirmed in the dark group since the upregulation in this group may have been probably limited the to much augmented plasma melatonin levels during the whole dark period since these levels were not 10-20 times higher than the control (just 2.5 folds higher than the control group) although they had been in whole dark during all the week.

With the down regulation of the receptors in the light period also supports our hypothesis where we suggest that it may limit the melatonin decrease up to zero-levels during a one week light exposure since these levels in our study were like the control levels. Because if it had been upregulated like the other hormones or like the melatonin do in other action sites, the Gi activation would be more and more on the pinealocyte membrane and the melatonin synthesis would be reached to zero-like level since the cAMP levels were declined so much. However, our findings suggest that by down regulating the melatonin receptor during the light in pinealocytes, it tries to maintain the Gi activation in controlled level which enables the Gs activation in a circadian pacemaker way regardless the light exposure therefore the plasma melatonin levels were found to be like as the control group not so much declined according to one week light exposure.

This hypothesis might explain the much lower MT1-receptor expression levels in the light group by down regulating the receptors, where plasma melatonin levels were found to be limited in the control level and also limited by upregulating the melatonin receptors probably leading the cAMP reduction from the exaggerated levels which we had been expecting in the dark group. We conclude that our report is the first report to show with Real-time PCR technology that pineal membrane has MT1 and MT2 receptors and the upregulation and down regulation of these receptors determine the plasma melatonin levels. Further studies on identifying the second messengers taking role in the melatonin synthesis and melatonin receptors use (like cAMP levels) would contribute to confirm this hypothesis.

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