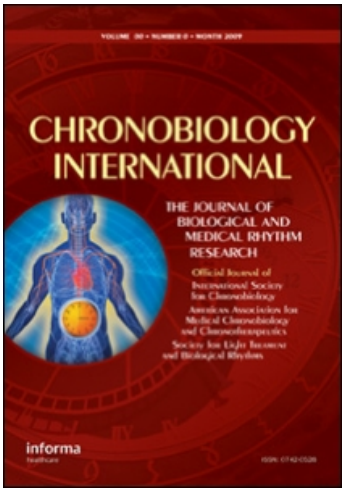

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RESPONSE OF MELATONIN RECEPTOR MT1 IN SPLEEN OF A TROPICAL INDIAN RODENT, *FUNAMBULUS PENNANTI,* TO NATURAL SOLAR INSOLATION AND DIFFERENT PHOTOPERIODIC CONDITIONS

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We analyzed the effect of natural solar insolation and artificial photoperiodic conditions on melatonin MT1 receptor expression of a tropical rodent, *Funambulus pennanti*. Melatonin mediates reproductive and circadian responses and regulates the production of a large number of cytokines, including interleukin-2 (IL-2), via modulation of MT1 receptor expression. Maximum pineal activity, resulting in high melatonin level, low melatonin receptor expression, and increased splenic mass, was noted in the winter months, while an opposite effect was noted during the summer months. Further, constant light exposure mimicked an "enhanced summer"-like condition with significant hyposplenia, and an opposite effect was observed with constant dark exposure with significant splenomegaly in *F. pennanti*. In the annual study, a slight increase in melatonin level was noted during the monsoon period, when the duration of photoperiod was the same but the amount of solar insolation and direct radiation decreased. The present study found that not only the duration of natural sunlight (i.e., photoperiod) but the intensity of sunlight expressed by solar insolation affects the circulatory level of melatonin and melatonin receptor expression in this wild tropical rodent. An increase in the circulatory level of melatonin induced a decrease in its receptor subtype MT1 expression in splenic cells, both at the transcriptional and translational levels, thus reflecting autoregulatory down-regulation of melatonin receptors. Therefore, in our animal model, *F. pennanti* melatonin may be suggested as a molecular messenger of photoperiodic signals (duration and intensity) directly acting via MT1 receptor regulation to adapt the immune system of animals residing in the tropical zone. (Author correspondence: chaldar2001@yahoo.com)

Keywords Melatonin, Melatonin receptors, Photoperiod, Solar insolation

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INTRODUCTION

The photoperiodic regulation of the melatonin rhythm makes the pattern of the hormone concentration an endocrine calendar for seasonally breeding animals (Haldar & Saxena, 1988a, 1988b; Pévet et al., 2002; Reiter, 1991, 1993). In the tropical zone, the duration of daylength in the summer and winter is comparatively shorter than in the temperate zone, but the annual cycle in rainfall, humidity, and amount of solar insolation plays a significant role in the regulation of annual/seasonal physiology. Melatonin (Pevet et al., 2002; Wen et al., 2006; Witt-Enderby et al., 2003), via its receptor types located on target tissues, is responsible for controlling these annual and daily events (Carrillo-Vico et al., 2003a, 2003b, 2005; Poirel et al., 2003; Skwarlo-Sonta et al., 2003). Monsoon is a typical and exclusive climatic condition experienced by the Indian subcontinent in the months of July and August. During this period, mostly dark clouds of cumulo-nimbus origin cover the sky (Gadgil, 2006; Haldar & Saxena, 1988a, 1988b). Although there is little change in the duration of the photoperiod, a difference has been recorded in the intensity of solar insolation and the amount of direct radiation and diffuse radiation reaching the earth's surface. Furthermore, the ecological conditions during monsoon are also quite harsh due to water logging, leading to lack of food and shelter for wild animals.

One of the most important non-neural targets of melatonin is the immune system (Hotchkiss & Nelson, 2002; Poon et al., 1994). Functional correlation between the pineal gland and immune system has been implicated by chronobiological studies of immune cell functions (Guerrero & Reiter, 2002; Haldar et al., 2001; Hotchkiss & Nelson, 2002). The existence of specific melatonin receptors may provide partial support for a direct effect of melatonin on the immune system (Carrillo-Vico et al., 2003b). Specifically, MT1 (IUPHAR, http://www.iuphar-db.org) shows a very prominent expression of mRNA and protein in rat thymus, spleen, and isolated T and B subsets of lymphocytes (Carrillo-Vico et al., 2003a; Poirel et al., 2003; Pozo et al., 1997; Skwarlo-Sonta et al., 2003). The earlier studies suggest a ubiquitous expression of the MT1 receptor in almost all tissues across species (Carrillo-Vico et al., 2003a; Poirel et al., 2003; Shiu et al., 2000; Skwarlo-Sonta et al., 2003). A lack of genetic data for *F. pennanti* led us to study in detail the melatonin rhythm and its correlation with the melatonin receptor MT1 expression rhythm (both at the mRNA as well as protein level) in an immunocompetent tissue (spleen) of the wild rodent, *Funambulus pennanti*, under two experimental setups. With Setup 1, we performed a two-year study divided into two-month periods of six equal parts. Under Setup 2, we noted the effect of three different photoperiodic conditions—constant light (24L:0D), equal light and dark (12L:12D), and constant dark (0L:24D) exposures—on immunity

and MT1 receptor expression to establish melatonin as the chemical messenger of light for immune regulation in *F. pennanti*.

MATERIALS AND METHODS

The experiments were conducted on adult male *F. pennanti* for two consecutive years, and the mean ± SEM of the data is presented. Fifteen squirrels approximately of same age (as judged by their cranium diameter, incisor length, and body weight [120 ± 5 gm]) were collected from the vicinity of Varanasi (latitude 25° 18′N and longitude 83° 1′E) in the first week of every month. Data for mean solar insolation were obtained from the NASA Surface Meteorology and Solar Energy, Atmospheric Data Center (http://eosweb.larc.nasa.gov).

One night at 22.00 h, prior to sacrifice, blood was collected under dim red light from the subclavian vein for RIA of melatonin. Following this, nine animals were sacrificed within a very short span of time (~5–10 min/animal) the next day at 11.00 h, and tissues were immediately weighed (Sartorius balance) and placed in liquid nitrogen for RT-PCR and Western blot analysis. Tissue was pooled every two consecutive months (based on almost equal solar insolation) for Western blot and RT-PCR studies.

To study the effect of experimental photoperiodic conditions, 45 adult male squirrels, approximately of same age and weight (120 ± 5 gm), were collected in the last week of September, weighed, and kept in wire net cages (25″ × 25″ × 30″) and exposed to 12L:12D and ambient temperature of 25 ± 2°C for acclimatization prior to exposure to different photoperiodic conditions. They were fed with soaked gram seed (*Cicer arietinum*), seasonal fruits, nuts, and water ad libitum. After 15 days of acclimatization (second week of October) to 12L:12D, the squirrels were randomized in the following three groups (n = 9): Group 1 was exposed to constant light (LL), Group 2 was exposed to 12L:12D, and Group 3 was exposed to constant dark (DD).

The photoperiodic exposures lasted 14 days. Food for the animals under the DD exposure was provided during the dark period of the comparable light-dark cycle with the help of a dim red light source. Because squirrels have a good olfactory sense of food, there was no effect on their health and body weight under DD. On the 14th day, at 23:00 h, blood was collected from the subclavian vein for RIA of melatonin. The following day, animals were sacrificed within a very short span of time. Spleens were immediately weighed. Tissues for RT-PCR and Western blot tissues were collected in liquid nitrogen.

All of the experiments were conducted in accordance with Institutional Practice and Guidelines of Committee for the purpose of Control and Supervision of Experimental Animals (CPCSEA) of the

Government of India, as well as in accordance with the ethical and methodological standards for laboratory and medical biological rhythm research (Portaluppi et al., 2008).

RNA Isolation

Total RNA was isolated using Trizol (Invitrogen, St. Louis, Missouri, USA) in accordance with the manufacturer's protocol. The RNA was precipitated with 1 volume of isopropanol and the pellet washed with cold 70% ethanol. The RNA thus obtained was intact and of good quality when tested by non-denaturing gel electrophoresis and spectrophotometric analysis.

Primers

Because the squirrel DNA sequences for melatonin receptor(s) and β-actin (as internal control) have not yet been determined, those for another rodent (i.e., the Wistar rat) were used. These primers were used by Poirel et al. (2003) to locate the MT1 receptor in a number of neural and non-neural tissues. The MT1 receptor amplification product is a single exon of 375 bp, and the β-actin primer is of 301 bp and spans an intron domain of 417 bp. The β-actin amplification product appears to be highly reproducible between samples, and independent experiments attest that the variation in MT1 amplification level seen between different experimental tissues is not due to variation in cDNA synthesis. Therefore, any change in amplification product at the end of PCR indicates the difference in amount of MT1 mRNA in the starting tissue sample. However, a positive PCR result could indicate genomic DNA contamination (Poirel et al., 2003). Hence, β-actin primer spanning an intron domain of 417 bp was selected (Poirel et al., 2003).

Further, to confirm the identity of the amplification product for the MT1 receptor primer, we performed PCR with genomic DNA and sequenced the amplification product with AB I3730 genetic analyzer (Certified GeneTool, Inc., USA) and submitted it to NCBI (accession no. FJ263047). The sequence has 78% identity with mouse MT1 receptor and 77% identity with rat and human melatonin MT1 receptor (Blastn, NCBI). The primers were synthesized by Bangalore Genei, India (see Table 1). The number of cycles were standardized at 40 for both the MT1 and β-actin primers.

RT-PCR Reaction

Reverse transcription reactions were performed for 1 h at 42°C using 1 μg of the total RNA in a 40 μl reaction mixture containing 5× MMLV

TABLE 1

Gene	Primer sequence 5′-3′	Size of product (bp) PCR	Size of product (bp) RT-PCR	Amplification profile 40 (cycles)
Mel 1a	TCACAATGGATGGAATCTGG (+) CACAGCAAGGCCAATGAAG (−)	375	375	94°C-1 min 58°C- 1 min 72°C- 1 min
β-actin	TGCTACATTTGCCACAGTCTCA (+) CCAGCACAGGGCAAAAAGTA (−)	718	301	94°C- 1 min 58°C- 1 min 72°C- 1 min

buffer, 0.5 mM dNTP mix, 0.5 μg Oligo d $(T)_{15}$ primer, 200 U of MMLV reverse transcriptase, and 40 U of RNasin ribonuclease inhibitor. All the reagents for the RT reaction were obtained from Promega (Madison, Wisconsin, USA).

The PCR reaction was performed in a 25 μl of reaction mixture, using 2.5 μl of the mix from the RT reaction, 10× Redtaq buffer, 8 μM of each of the primers, 0.2 mM dNTPmix, and 1.5 units of Redtaq polymerase (Sigma, St. Louis, Missouri, USA). Polymerase chain reaction was performed in a thermocycler (MJ Research, Watertown, Massachusetts, USA). The amplification condition for each of the primers is listed in Table 1, with 40 cycles. Each PCR reaction started with 3 min denaturation at 94°C and ended with 20 min of synthesis at 72°C (Poirel et al., 2003).

Electrophoresis

10 μl of the PCR products were analyzed by electrophoresis on a 1.5% agarose gel in TBE buffer, according to standard techniques (Sambrook et al., 2001). The gel was stained with ethidium bromide (0.5 μg/ml), and the DNA bands were visualized with a UV-transilluminator and scanned with an image analyzer (AlphaImager, Alpha Innotech, USA). Direct Load ™ Wide Range DNA marker (Sigma, D7058) was used to evaluate the size of the amplification product. The whole process was repeated three times with three independent lots of tissues. An independent series was treated with Rnase free Dnase (Bangalore Genei, India) prior to cDNA synthesis to clearly demonstrate that the observed amplification products derived from actual tissue MT1 mRNA expression and not from genomic DNA. All of the results are expressed as mean ± SEM.

Western Blot Analysis

At the end of the photoperiodic exposure and for the annual experiment, squirrels from each group were sacrificed. The tissues were

removed immediately on ice, weighed, and placed in ice-cooled glass homogenizers, and a 10% homogenate was prepared using lysate buffer (1% NP40, 0.1% aprotonin, 20 μl of 50 mM sodium orthovanadate, and 1% PMSF in PBS). After centrifugation at 13,000 g for 10 min, the supernatant was estimated for protein concentration by Bradfords Kit (Amersham, U.K.).

10 μg samples were run on a 12% reducing SDS-polyacrylamide gel along with Rainbow marker (Amersham, RPN480). For immunoblot studies, the protein was electrotransfered (Fast Blot, Biometra) to PVDF membrane followed by immunodetection with MT1 antibodies. MT1 antibody is affinity purified and raised in goat against a peptide mapping the amino terminus and has been previously used in rodent models (37.5kDa; goat IgG, SC13179, Biotech, Santa Cruz, California, USA; see Lahiri et al., 2006; Shiu et al., 2000; Treeck et al., 2006) and detected using the chemiluminisence (ECL) system (Amersham, UK). Further, the PVDF membranes were stripped with stripping buffer (10% sodium azide) and immunostained with β-actin antibodies (42kDa; Sigma A1978, USA) and used as an internal control.

Densitometry

Bands obtained after ECL detection were scanned and their intensities quantified using Scion image analysis software (http://www.scioncorp.com). The mean ± SEM were calculated from the data obtained from three independent sets of experiments after normalization with β-actin and calculation of the relative intensity values.

RIA of Plasma Melatonin

RIA of melatonin was done following the method of Attanasio et al. (1985). A nighttime (22:00 h) blood sample was collected from the subclavian vein for RIA. Validation of the radioimmunoassay was performed as described earlier by Bishnupuri and Haldar (2001) and Haldar et al. (2001, 2004) for this animal model. 3H-melatonin (Amersham, UK) and sheep anti-melatonin (Stockgrand, UK) were used for the assay. The intra-assay and inter-assay CVs were 9 and 15%, respectively. Percent recovery after extraction was 92%. The sensitivity of the assay was ±10 pg/ml for 200 samples, and the cross-reactivity of the antibody with melatonin and 5-MT antigens was 1.00% and 0.05%, respectively.

Statistics and Calculations

Analysis of variance (ANOVA) and Newman-Keuls test with GraphPad Prism-software were used to determine the level of significance. Statistical

significance was defined as $p < 0.001$ and n = 9. The mean ± SEM was calculated from the data from three independent sets of experiments. To study annual variation in solar insolation and its effect on splenic weight, data were pooled and averaged for every two-month period, dividing the whole year into six periods. For reverse transcriptase-PCR and Western blot, the mean ± SEM was calculated per experimental photoperiod using data obtained from three independent sets of experiments after normalization with β-actin. Calculated relative intensity values are presented as a percent expression. To study the annual variation in solar insolation and its effect on MT1 receptor expression by reverse transcriptase-PCR and Western blot, tissues were pooled every two consecutive months and the mean ± SEM was calculated after normalization with β-actin and calculation of the relative intensity values.

RESULTS

Effect on Splenic Weight

Animals showed a decrease in relative splenic weight (mg/100 g body weight) as the amount of solar insolation increased from 4.105 kWh/sqm/day in the months of November–December to 6.19 kWh/sqm/day in the months of May–June. The relative spleen weight in the months of May–June was the lowest, while the relative spleen weight in the months of November–December was the highest (see Figure 1b; $p < 0.001$; n = 9). Morphologically, we also observed a distinct difference in the size of the spleen collected from animals exposed to LL and DD conditions (see Figure 2a). The spleen weight of animals housed under DD was increased significantly in comparison to controls housed under 12L:12D (see Figure 2b; $p < 0.001$; n = 9). In contrast, spleens of animals housed under LL showed a significant decrease in weight compared to control animals housed under 12L:12D (see Figure 2b; $p < 0.001$; n = 9).

Serum Melatonin Level

When the serum level of melatonin was recorded for one year under six periods of almost equal solar insolation, a significantly lower level was observed during the months of May–June (see Figure 1a; $p < 0.001$; n = 9) and a higher level during the months of November–December, with a small secondary peak in July–August (see Figure 1a). The serum level of melatonin of the squirrels was significantly decreased (see Figure 2c; $p < 0.001$; n = 9) under LL in comparison to the control 12L:12D condition, while under DD it increased significantly compared with control the condition (see Figure 2c).

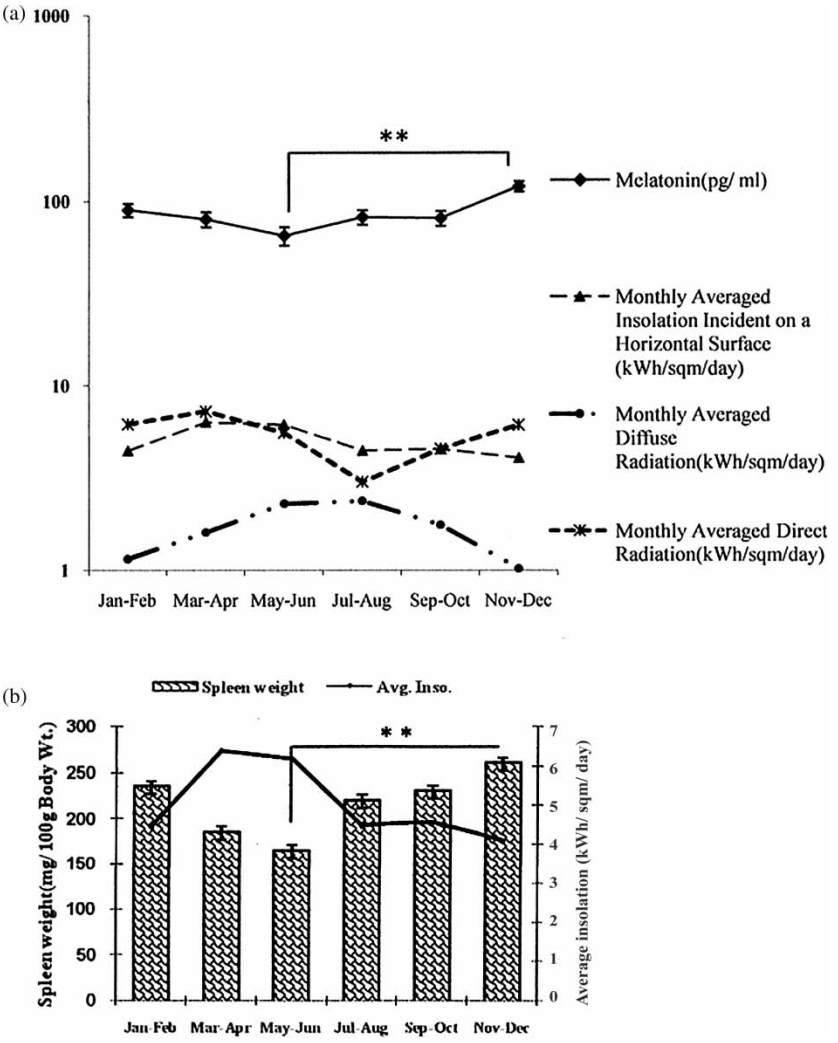

FIGURE 1 Amount of annual averaged solar insolation, diffused radiation, direct radiation for every two monthly period and effect on (1a) circulatory melatonin level (pg/ml) and (1b) spleen weight (mg/100 g body wt) in *F. pennanti*. Data shown are mean ± SEM of mg/100 g body wt for splenic weight and pg/ml for serum level of melatonin; $p < 0.001$; n = 9.

Expression of Melatonin Receptor (MT1) mRNA and Protein on T-Helper Cells of the Spleen under Different Periods of Solar Insolation

A significantly higher ($p < 0.001$; n = 9) level of both MT1 mRNA and protein was observed in the months of May–June, when the level of solar insolation was at its second highest (6.19 kWh/sqm/day) peak (see Figures 3a and 3b). The lowest level of both MT1 mRNA and

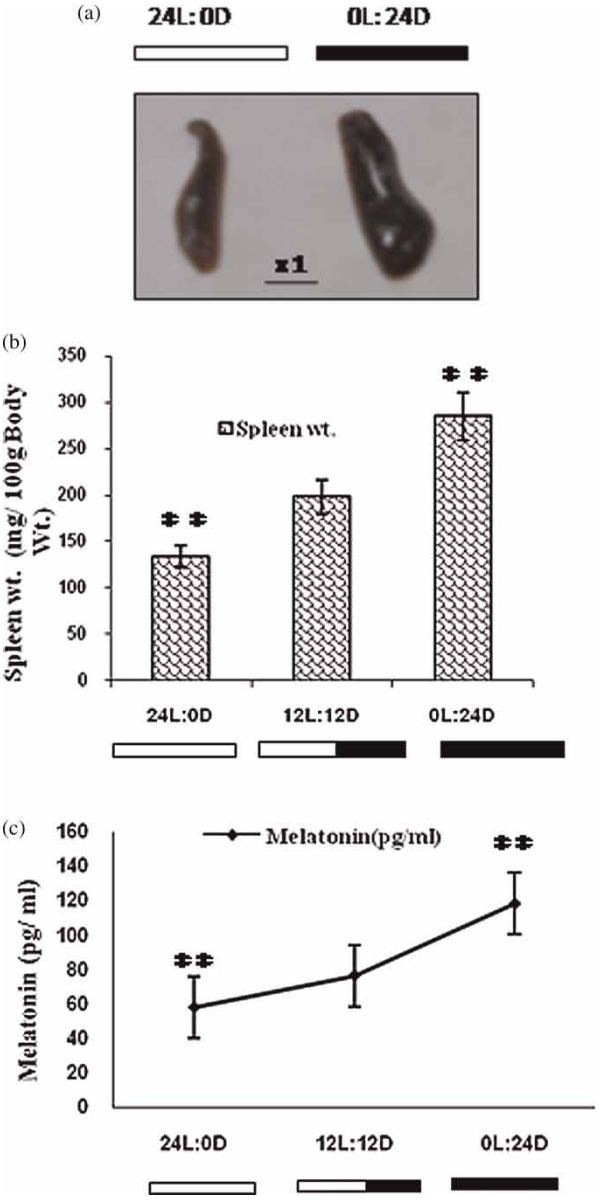

FIGURE 2 Effect of constant light (24L:0D), equal light and dark (12L:12D), and constant dark (0L:24D) exposure on (2a) visible size and (2b) weight of spleen, and (2c) serum level of melatonin of *F. pennanti*. Data shown are mean ± SEM of mg/100 g body wt for splenic weight and pg/ml for serum level of melatonin; $p < 0.001$; n = 9.

protein was noted from November to February. The rest of the time periods showed a gradual decrease, except in the months of July–August, when there was a significant decrease ($p < 0.001$; n = 9) in both MT1 mRNA and protein (see Figures 3a and 3b).

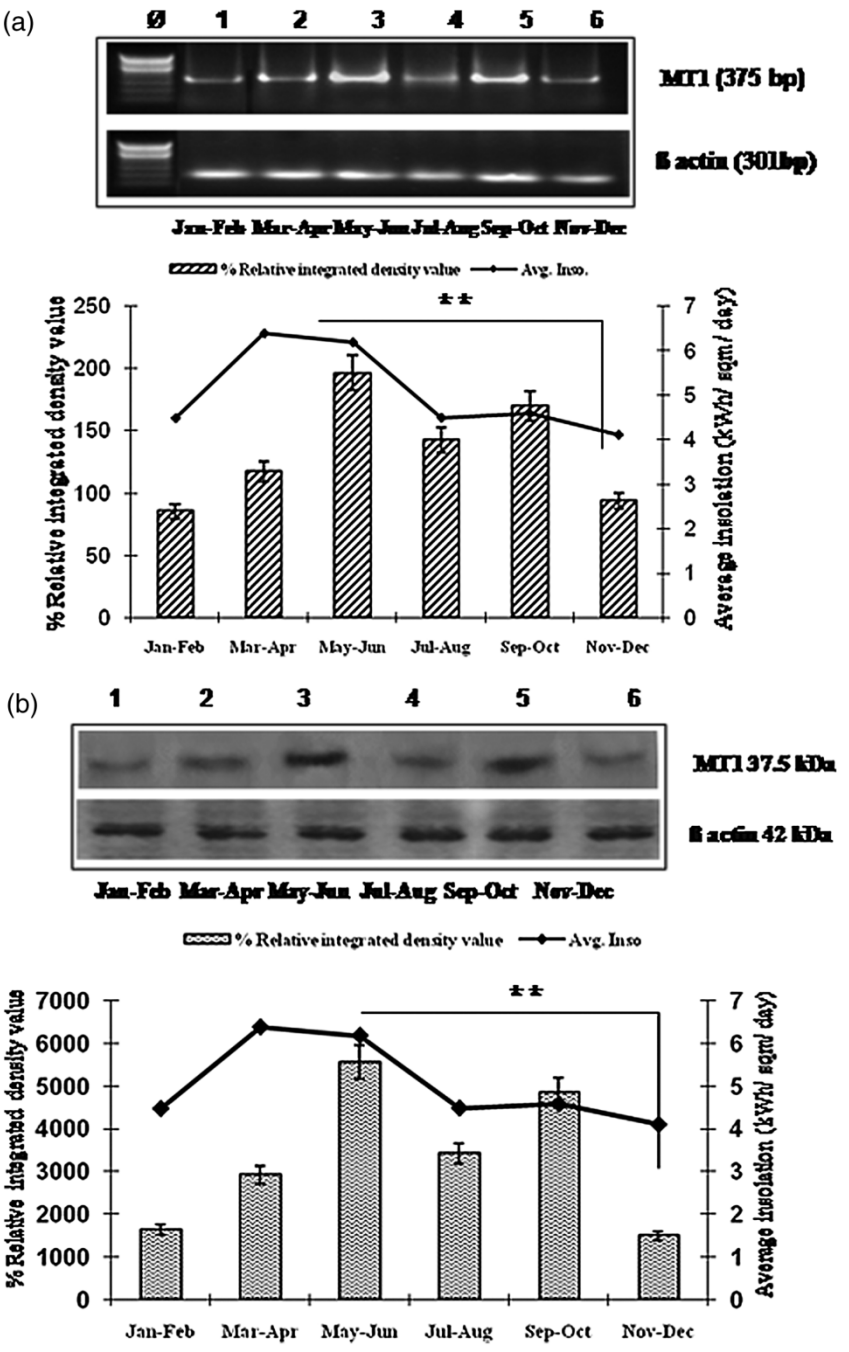

FIGURE 3 Effect of annual average solar insolation on expression of membrane melatonin receptor (MT1) (3a) mRNA (375 bp) and (3b) protein (37.5kDa) and β actin (42 kDa) expression on T-helper cells of spleen in *F. pennanti*. Data shown are mean ± SEM of % relative integrated density value after normalization with β actin; $p < 0.001$; n = 9.

Effect of LL and DD Treatment on Melatonin Receptor (MT1) mRNA and Protein Located on Splenocytes

To determine the effect of LL and DD treatment on melatonin receptor (MT1) mRNA and protein located in the spleen of *F. pennant*, we considered the level of expression in 12L:12D treated control animals as reference (100%). DD significantly down-regulated MT1 mRNA and protein level ($p < 0.001$; n = 9) compared to the control (12L:12D) situation (see Figures 4a and 4b). LL significantly up-regulated both MT1 mRNA and protein ($p < 0.001$; n = 9; see Figures 4a and 4b).

DISCUSSION

A well-known relationship exists between photoperiodism and immunity (Ahmad & Haldar, 2009; Giordano et al., 1993; Maestroni, 1993; Skwarlo-Sonta et al., 2003) establishing an interesting dimension of research that may be designated as photoimmunomodulation. Over the past 30 yrs, animal studies have shown that environmental light is the primary stimulus for regulating circadian rhythms, seasonal cycles, and neuroendocrine responses (Aschoff, 1981b; Binkley, 1990; Cardinali et al., 1972). With all photobiological phenomena, the wavelength, intensity, timing, and duration of a light stimulus are important in determining the regulatory influence on circadian and neuroendocrine systems (Aschoff, 1981a; Brainard et al., 1983, 1986; Cardinali et al., 1972; Schuster et al., 2001) via its regulatory influence on the pineal gland to effect melatonin synthesis (Binkley, 1990, Recio et al., 1998; Wen et al., 2006). Enhancement of immune response was previously suggested to be dependent on photoperiod and melatonin (Haus & Smolensky, 1999; Hotchkiss & Nelson, 2002; Pozo et al., 1997). The relationship between annual rhythms in melatonin and immune system status in *Funambulus pennanti* has previously been established (Haldar & Saxena, 1988b; Haldar et al., 2001).

Previous studies also indicate that lymphocytes synthesize melatonin (Carillo-Vico et al., 2004), and there is ubiquitous expression of MT1 receptor mRNA and protein in T and B subsets of lymphocytes in the spleen and thymus of the rat and mouse, unlike the MT2 receptor (Carillo-Vico et al., 2003a; Pozo et al., 1997; Skwarlo-Sonta et al., 2003). In *Phodopus sungorus*, well known as "nature's knockout" for the MT2 receptor, the MT1 receptor mediates reproductive and circadian responses to melatonin (Schuster et al., 2001; Weaver et al., 1996) and the inhibitory effect of prostaglandin E_2 on IL2 production in human lymphocytes (Carrillo-Vico et al., 2003a). In the present study, we have noted the effect of experimental and natural photic and photoperiodic conditions on the expression of the membrane melatonin receptor MT1

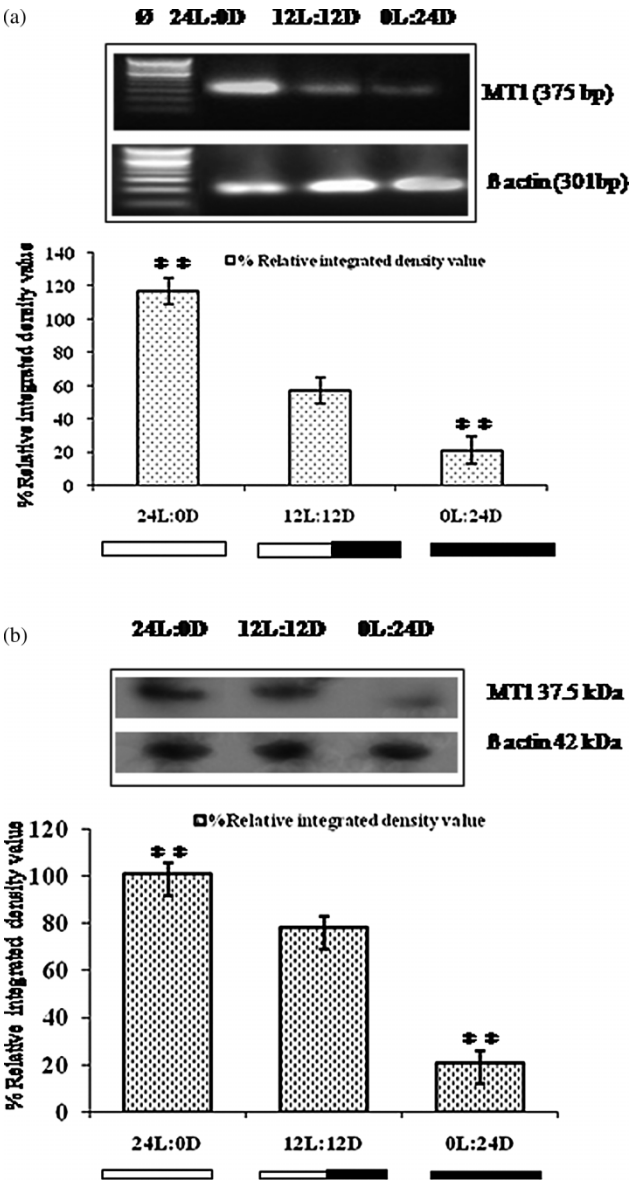

FIGURE 4 Effect of constant light (24L:0D), equal light and dark (12L:12D), and constant dark (0L:24D) exposure on (4a) mRNA (375 bp) and (4b) protein (37.5 kDa) expression and β actin (42 kDa) expression on T-helper cells of spleen in *F. pennanti*. Data shown are mean ± SEM of % relative integrated density value after normalization with β actin; $p < 0.001$; n = 9.

located on splenocytes. For this, we studied the change in expression of the MT1 melatonin receptor (MT1) mRNA and protein located on spleen cells of *F. pennanti*. Interestingly, we found both melatonin receptor (MT1) mRNA and protein showed a significant decrease after DD exposure and an increase following LL exposure. This indicates a possible

role of light exposure, relative to the control 12L:12D condition in modulation of immunity by regulating MT1 membrane receptor expression in our animal model *F. pennanti*.

The findings of the present study parallel previous ones derived from studies on Siberian and Syrian hamsters, where the regulation of MT1 mRNA and protein by the photoperiod directly depends on the RNA transcription level (Masson-Pévet et al., 2000; Schuster et al., 2001). Melatonin binding and receptor mRNA levels are directly affected by light and plasma melatonin concentration in rodents (Gauer et al., 1993a, 1993b; Masson-Pévet et al., 2000; Recio et al., 1996; Reppert et al., 2000; Schuster et al., 2001). Autoregulatory down-regulation of the melatonin receptor MT1 expression has been suggested by Barrett et al., (1996). In the rodent suprachiasmatic nucleus and Pars Tuberalis, MT_1 mRNA expression and ^{125}I-melatonin binding exhibit daily variations, with elevated levels occurring during the daytime (Masson-Pévet et al., 2000; Recio et al., 1998; Reppert et al., 2000; Schuster et al., 2001). Light exposure during the night also increases ^{125}I-melatonin binding, coinciding with the suppression of melatonin synthesis (Masson-Pévet et al., 2000; Recio et al., 1998; Reppert et al., 2000), indicating melatonin down-regulates its receptor population (Barrett et al., 1996; Gauer et al., 1993b). Other studies involving manipulation of melatonin levels also support the view the hormone regulates its own receptors (Recio et al., 1998; Schuster et al., 2001), as we noted in the present study with different photoperiodic exposure.

Increase in solar insolation by 2.09 kWh/sqm/day resulted in an increase in expression of MT1 mRNA and protein in *F. pennanti*. During the months of May–June, a small decrease was noted in the amount of solar insolation (0.05 kWh/sqm/day) and thereby direct radiation due to an approaching South-West monsoon, but the amount of diffuse radiation remained high; this might have helped the squirrels maintain a high level of MT1 mRNA, as the clouds were not stagnant. Our suggestion is supported by various reports that different types of radiation influence melatonin level in vivo and in vitro (Brainard et al., 1986; Griefahn et al., 2006; Kassayova et al., 1995; Saito et al., 2000). Nonetheless, in the peak monsoon period (i.e., in the months of July–August), there was a drastic reduction in the amount of solar insolation, from 6.24 to 4.49 kWh/sqm/day, due to the dark monsoon clouds of cumulo nimbus origin (Gadgil, 2006), that was approximately equivalent to the amount of solar insolation experienced in the winter months (i.e., November–December), though the duration of the photoperiod remained almost the same. Interestingly, this decrease induced a direct decrease in transcription and translation of the MT1 receptor gene. This level again rose slightly by 0.09 kWh/sqm/day in the months of September–October and finally decreased significantly by the months of

November–December to 4.105 kWh/sqm/day. This could be the reason that photoimmunomodulation (and/or photoperiodic regulation of melatonin level and immunity) has become a popular approach in clinical science (Nelson, 2004). Earlier, studies indicated that melatonin exerts its seasonal effects on the pars tuberalis by signaling specific cells through the MT1 receptor subtype (Gauer et al., 1993a, 1993b; Reppert et al., 2000). In the present study, we found that the amount of solar insolation in terms of kWh/sqm/day showed a direct relationship with the circulatory level of melatonin, splenic weight, and MT1 receptor mRNA and protein expression of T-helper cells of the spleen in *F. pennanti*, which could be of high adaptive significance for the survival of wild animals inhabiting tropical regions and experiencing a monsoon-type of climate, thereby protecting them from monsoon-related seasonal diseases (e.g., dermal and eye infections).

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The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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